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Award Number: W81XWH-04-1-0009

TITLE: GKLF as a Novel Target in Selenium Chemoprevention of Prostate Cancer

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Buffalo, NY 14263

February 2008

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-02-2008	Final Addendum	1 Feb 2007 – 31 Jan 2008

4. TITLE AND SUBTITLE

GKLF as a Novel Target in Selenium Chemoprevention of Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0009

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Roswell Park Cancer Institute
Buffalo, NY 14263

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The present study investigated the functional significance of the zinc finger transcription factor gut-enriched krüppel-like factor (GKLF) in mediating selenium action in the androgen receptor (AR)-null PC-3 human prostate cancer cells. We found that overexpression of GKLF enhances selenium inhibition of DNA synthesis and induction of apoptosis. Furthermore, knocking down the expression of GKLF greatly attenuates the growth suppressive and apoptosis inducing activities of selenium. Therefore, our data support an important role of GKLF induction in selenium action in the AR-null prostate cancer cells. However, we found that, in cells expressing a functional AR, the disruption of AR signaling is most likely more important than the induction of GKLF signaling for selenium action. Selenium treatment significantly decreases the expression of AR and AR-regulated genes implicated in prostate carcinogenesis (PSA, KLK2, ABCC4, DHCR24, and GUCY1A3) in five human prostate cancer cell lines irrespective of their AR genotype (wild-type vs. mutant) or sensitivity to androgen-stimulated growth. Transfection of AR in the androgen-dependent LNCaP cells weakens significantly the inhibitory effect of selenium on cell proliferation and AR target gene expression. Since the vast majority of prostate cancers, including those refractory to hormone therapy, express a functional AR, the disruption of AR signaling is probably more important for selenium action and more relevant to selenium chemoprevention of prostate cancer than the induction of GKLF.

15. SUBJECT TERMS
selenium, cancer chemoprevention, prostate cancer, GKLF transcription factor, androgen receptor

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	30	19b. TELEPHONE NUMBER *(include area code)*

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Molecular Cancer Therapeutics, 4, 1047-1055, 2005.
Molecular Cancer Research, 6, 306-313, 2008

A. INTRODUCTION:

This project was designed initially to investigate the role of the zinc finger transcription factor gut-enriched krüppel-like factor (GKLF) in contributing to the molecular effects of selenium in cancer chemoprevention. In the first annual progress report, we described the mechanistic basis for GKLF upregulation by selenium and the effect of GKLF overexpression on the growth of prostate cancer cells. Our data indicated a growth suppressive and pro-apoptotic function of GKLF in the androgen receptor (AR)-null PC-3 cells. However, the LNCaP cells, which contain a functional AR, responded to GKLF overexpression by inducing the expression of AR, and the effect of which predominated, leading to a modest stimulation of cell growth. We also found that selenium is able to markedly suppress AR expression. Exogenous expression of AR attenuated the growth suppressive activity of selenium, although accompanied by a significant increase in GKLF level. The data suggest that disruption of AR signaling is probably more important than the induction of GKLF signaling for selenium action in AR-expressing cells. Therefore, as approved by the DOD Prostate Cancer Research Program, we continued our GKLF study in the AR-null PC-3 cells, but shifted our research focus to selenium suppression of AR signaling in the androgen-responsive LNCaP cells.

B. BODY:

Results for Task 1 (To study whether transcriptional or post-transcriptional mechanism is responsible for mediating selenium upregulation of GKLF expression):

Key findings (please see the attached article (Liu, *et al.*, Mole. Cancer Res., 6, 306-313, 2008) in the Appendix for detailed descriptions:

- MSA induces GKLF mRNA level.
- MSA increases the stability of GKLF mRNA.
- MSA increases GKLF transcription initiation.
- MSA Induces the DNA-Binding Activity of GKLF.

Results for Task 2 (To determine the effect of GKLF overexpression on the growth of prostate cancer cells as well as selenium growth inhibition):

Key findings (please see the attached article (Liu, *et al.*, Mole. Cancer Res., 6, 306-313, 2008) in the Appendix for detailed descriptions:

- Overexpression of GKLF enhances MSA inhibition of DNA synthesis in PC-3 cells.
- Overexpression of GKLF induces apoptosis and enhances the effect of MSA on apoptosis induction in PC-3 cells.

Results for Task 3 (To assess the effect of GKLF gene knockdown on the growth inhibitory and apoptosis inducing actions of selenium):

Key findings (please see the attached article (Liu, *et al.*, Mole. Cancer Res., 6, 306-313, 2008) in the Appendix for detailed descriptions:

- GKLF knockdown weakens the growth suppressive activity of MSA in PC-3 cells.
- GKLF knockdown attenuates the apoptosis induction activity of MSA in PC-3 cells.
- GKLF knockdown attenuates the effect of MSA on the modulation of GKLF target genes in PC-3 cells.

Results for Task 4 (To investigate the effect of androgen receptor overexpression on the growth inhibitory and gene-expression modulating activities selenium):

Please see the attached article (Dong *et al*., Molecular Cancer Therapeutics, 4, 1047-1055, 2005) in Appendix for detailed description of the specific aspects of the research pertinent to this task.

Results for Task 5 (To characterize the mechanism(s) by which selenium suppresses androgen receptor signaling):

Inhibition of AR transcription initiation by MSA. To determine whether MSA-mediated downregulation of AR was due to increased mRNA degradation or decreased transcription, we performed an mRNA stability assay. Our results showed that treatment with 10 μM MSA slightly increased the stability of AR mRNA (Fig. 1A).

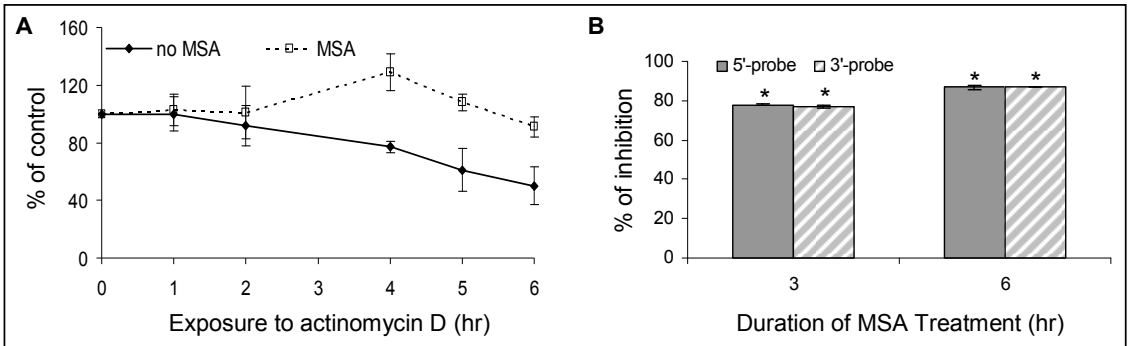

Fig. 1. A, Effect of MSA on AR mRNA stability. The mRNA level was quantified by real-time RT-PCR. B, Suppression of AR transcription initiation by 10 μM MSA as assessed by real-time RT-PCR of nascent RNA obtained by run-on transcription. The real-time RT PCR analysis was conducted with a primer-probe set corresponding to either the 5'-end or the 3'-end of the AR mRNA. *, statistically different compared to untreated control ($P < 0.05$).

We then studied the effect of MSA on AR transcription by nuclear run-on assay. Biotin-labeled nascent transcripts obtained by run-on transcription were isolated by using streptavidin particle beads, and quantitated by real-time RT-PCR. To differentiate the effect of MSA on transcription initiation and on transcription elongation, the real-time RT-PCR analysis was conducted with a primer-probe set corresponding to either the 5'-end or the 3'-end of the AR mRNA. For both sets of primer-probe, MSA treatment resulted in ~80% and 87% inhibition of AR transcription at 3 hr and 6 hr, respectively (Fig. 1B). The data thus indicate that the decrease in AR mRNA level by MSA is accounted for by a vigorous block of AR transcription initiation.

Inhibition of AR promoter activity by MSA. Gene transcription is generally controlled by promoter regions. In order to study the effect of MSA on AR promoter activity, we PCR amplified an 8 kb and a 1.7 kb fragment of the 5'-flanking region of the human AR gene from an AR-containing BAC clone. The 8 kb fragment (-6885 to +1115) contains ~6.9 kb of the 5'-flanking region upstream of the entire 5'-untranslated region (UTR), and the 1.7 kb fragment (-600 to +1115) contains 600 bp of the 5'-flanking region upstream of the entire 5'-UTR. These two promoter regions were then cloned into the pGL4.19[*luc*2CP/Neo] rapid response luciferase expression vector (Promega). The inclusion of the protein degradation sequences in this vector allows the reporter to be highly responsive and suitable for monitoring rapid response. The

authenticity of the constructs, *pGL4-8kb-AR-promoter and pGL4-1.7kb-AR-promoter*, was confirmed by DNA sequencing. The two constructs were transiently transfected into LNCaP cells cultured in a defined medium (RPMI1640 plus 2% albumax) without androgen to avoid the confounding effect of AR auto-regulation. The treatment duration was 1 hr, at a time when there was no detectable decrease of AR protein. As shown in Fig. 2, MSA induced a ~60% inhibition of the activity of both promoters. The data therefore indicate that the *cis* element(s) mediating MSA downregulation of AR is present in the 1.7 kb promoter region.

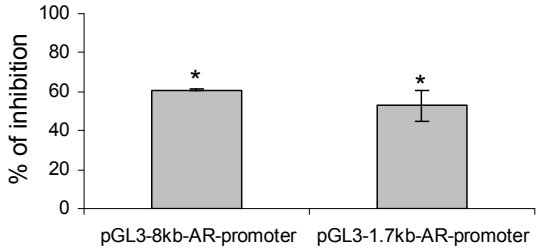

Fig. 2. Suppression of AR promoter activity by 10 μM MSA as assessed by luciferase assay. *, statistically different compared to untreated control ($P < 0.05$).

No change of AR ligand-binding by MSA. It is generally believed that unliganded AR localizes in the cytoplasm as a heteromeric complex with heat shock proteins (1;2). Upon binding to androgens, AR undergoes conformational change, dissociates from the heteromeric partners, forms a homodimer through the interaction of AR N- and C-terminal regions, and translocates into nucleus to initiate target gene regulation (1;2). In addition, the *trans*-activating activity of AR is modulated by other transcription factors and coregulators (3). Our previous data suggest that MSA may disrupt AR signaling through additional mechanism(s) beyond reducing the availability of the AR protein. We therefore assessed the effect of MSA on the ligand-binding activity of AR.

The ligand-binding activity of AR was analyzed in a whole-cell radioligand binding assay. LNCaP cells were incubated in medium containing charcoal-stripped serum. Increasing concentrations (0.0094, 0.0188, 0.0375, 0.075, 0.15, and 0.3 nM) of [^{3}H] R1881 were added to the culture at the time of 10 μM MSA treatment, with or without a 200-fold molar excess of unlabeled R1881. The radioactivity was measured at the 2-hr time point when there was no change in level of the AR protein. The binding capacity and affinity were determined by Scatchard plot analysis. The values of Bmax (the number of binding sites) and Kd (the concentration of [^{3}H] R1881 producing 50% of total receptor occupancy) are shown in Fig. 3. It is apparent that MSA treatment did not alter the binding capacity or affinity of AR.

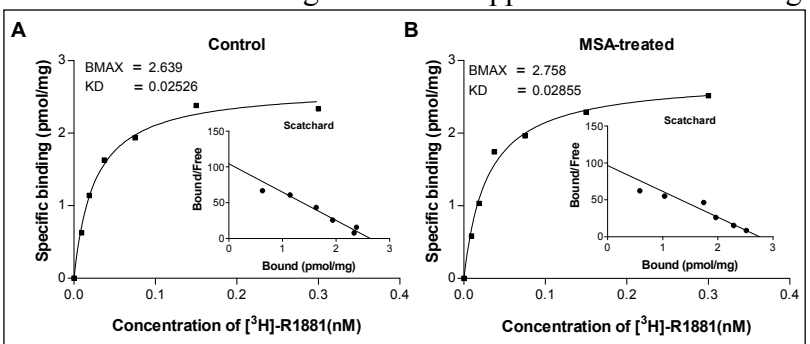

Fig. 3. No effect of MSA on AR ligand-binding activity as assessed by the whole-cell radioligand binding assay. LNCaP cells were treated with 10 μM MSA for 2 hr, at a time when there was no change in level of the AR protein. Bmax and Kd represent the number of binding sites and the concentration of [^{3}H] R1881 producing 50% of total receptor occupancy, respectively.

Suppression of AR N-C dimerization by MSA. The effect of MSA on AR N-C dimerization was assessed by using a mammalian two-hybrid system kindly provided by Dr. Elizabeth M. Wilson at the University of North Carolina. As illustrated in Fig. 4A, this system includes two fusion protein constructs, VP-A1 and GALD-H, as well as one reporter gene plasmid, G5E1bLuc (4). VP-A1 is the fusion construct of the N-terminal residues 1-503 of AR

and the activation domain of the herpes simplex virus VP16 protein. GALD-H is the fusion construct of the C-terminal ligand-binding domain of AR (624-919) and the GAL4 DNA-binding domain. The G5E1bLuc construct contains the luciferase reporter gene downstream of five consensus GAL4 binding sites and the minimal promoter of the adenovirus E1b gene. Once the three constructs are co-transfected into cells, the interaction of the AR N- and C-terminal regions brings together the DNA-binding and transactivation functions, leading to the expression of the reporter gene. Since endogenous AR interferes with the interaction of the two fusion proteins, we transfected the mammalian two-hybrid system into the AR-null PC-3 human prostate cancer cell line. Cells were exposed to 10 μM MSA and/or 10 nM R1881 for 3, 6, or 16 hr. As shown in Fig. 4B, exposure to R1881 resulted in AR N-C dimerization, which was greatly suppressed by MSA.

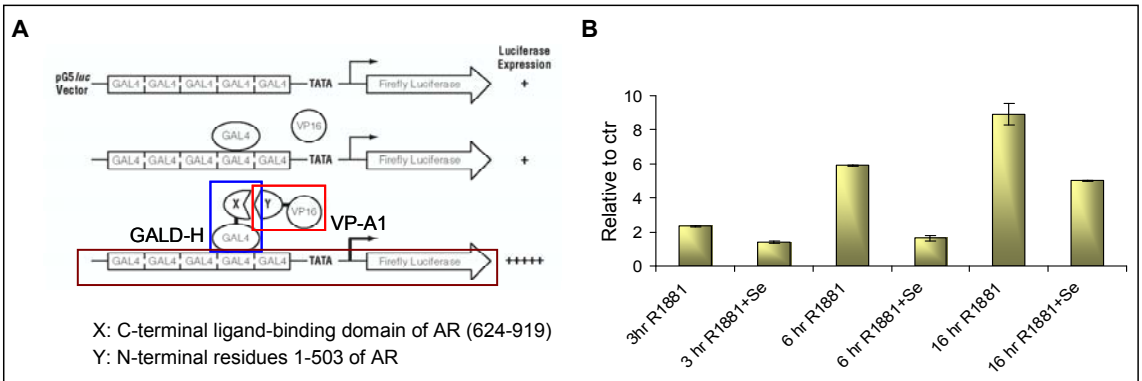

Fig. 4. Suppression of on AR N-C dimerization by MSA. PC-3 cells transfected with a mammalian two-hybrid system were exposed to 10 μM MSA and/or 10 nM R1881 for 3, 6, or 16 hr. The data are presented as fold of change compared to the vehicle control (no MSA or R1881).

<u>Modulation of an AR-coregulator, HNF3α, by MSA.</u> Our previous bioinformatic data mining analysis of microarray data has alerted us to a potential role of hepatic nuclear factor 3α (HNF3α, also known as forkhead box A1 or FOXA1) in MSA action (5). HNF3α is a member of the forkhead family of transcription factors. HNF3α physically interacts with AR (6). Two functional HNF3-binding elements (HBEs) are located adjacent to the AREs in the core enhancer region of the PSA gene (6). A similar organization of HBEs and AREs are also present in the promoter of 2 additional prostate-specific genes, probasin (rat) and prostatic acid phosphatase (6). HNF3α binding to the HBEs is essential for maximal androgen induction of PSA and probasin (6). Our data mining analysis identified HNF3α as one of the top 50 genes upregulated in prostate cancer (5), and more importantly, MSA downregulates the expression of HNF3α (5).

I. MSA inhibition of HNF3α expression and DNA-binding activity. We performed real-time RT-PCR analysis to confirm the modulation of HNF3α by MSA in LNCaP and LAPC-4 cells. As shown in Fig. 5A, a ~65% reduction of HNF3α transcript was detected as early as 3 hr after exposure to 10 μM MSA; the magnitude of inhibition rose to ~80% by 6 hr. Alteration in HNF3α protein preceded the change in HNF3α mRNA. A 50% reduction in the level of HNF3α protein was evident at 1 hr post MSA (Fig. 5B). The effect of MSA on HNF3α seemed to begin with a profound inhibition of protein stability, and transcriptional or post-transcriptional regulation kicked in later. We next quantified the DNA-binding activity of HNF3α by using the TransAM HNF assay kit (Active Motif), which is an ELISA-based EMSA system. The data were consistent with the protein results. A 25% depression of HNF3α DNA-binding activity

was detectable as early as 1 hr post MSA, and the inhibition reached to ~35% at 3 hr (Fig. 5C). The wild-type competitor oligo almost completely abolished the binding, whereas the mutant oligo produced no effect, thus demonstrating the specificity of the interaction.

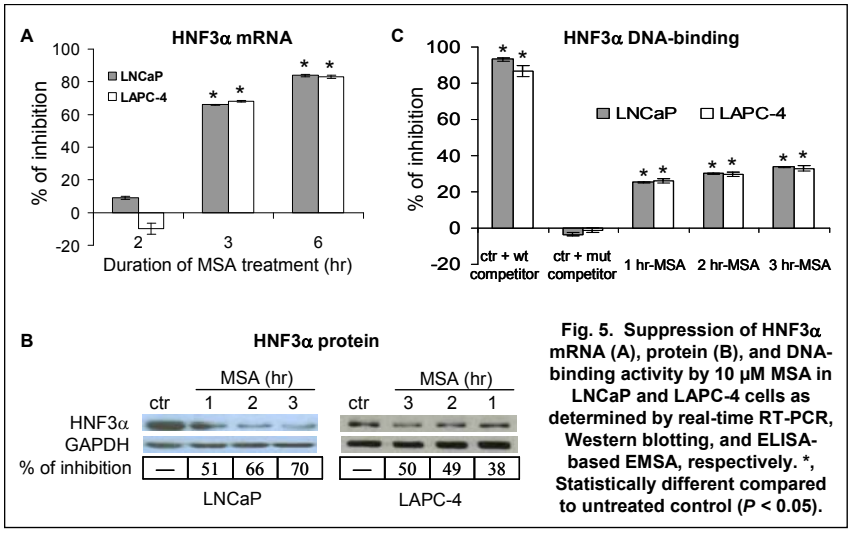

Fig. 5. Suppression of HNF3α mRNA (A), protein (B), and DNA-binding activity by 10 μM MSA in LNCaP and LAPC-4 cells as determined by real-time RT-PCR, Western blotting, and ELISA-based EMSA, respectively. *, Statistically different compared to untreated control (*P* < 0.05).

II. Potential involvement of HNF3α in MSA suppression of PSA promoter activity. In order to determine whether the binding of HNF3α to the two HBEs located adjacent to the AREs in the core enhancer region of the PSA gene is involved in MSA suppression of PSA transcription, we transiently transfected LNCaP cells with 3 PSA promoter-enhancer reporter constructs, PSA-EP, mPSA-EP1 and mPSA-EP2. These constructs were kindly provided by Dr. Robert Matusik at the Vanderbilt University. PSA-EP contains an 823-bp enhancer fragment (-4758 to -3935 nt) upstream of the PSA minimal promoter (-610 to +11 nt) in the pGL3-basic vector (6). mPSA1-EP and mPSA2-EP are two mutant PSA-EP constructs containing mutations in the HBEs (6). The cells were cultured in medium containing charcoal-stripped serum. As shown in Fig. 6, R1881 treatment resulted in a marked induction of PSA enhancer-promoter activity, and the induction was significantly blocked by 10 μM MSA. Mutations in the two HBEs not only greatly compromised R1881 induction of PSA enhancer, but also significantly attenuated MSA inhibition of the activity of the PSA enhancer-promoter.

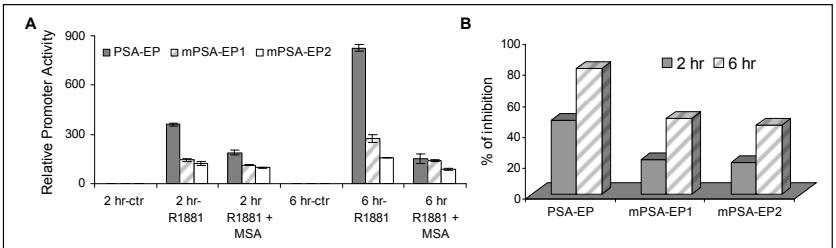

Fig. 6. Mutation of HBE in PSA enhancer-promoter compromised androgen-induction of as well as MSA-inhibition of PSA enhancer-promoter activity. PSA-EP is a wild-type PSA promoter-enhancer reporter gene construct, and mPSA-EP1 and mPSA-EP2 are two mutant constructs containing mutations in HBE. Results in A are presented as relative to the untreated control for PSA-EP at the respective time point. Results in B are presented as % of inhibition by MSA as compared to the respective R1881-treated sample.

III. Enhancing MSA action by HNF3α knockdown. To further confirm the involvement of HNF3α in MSA modulation of AR target genes, we transiently transfected LNCaP cells with a Stealth HNF3α siRNA duplex (Invitrogen) and assessed the response of the HNF3α-knockdown cells to MSA downregulation of PSA and KLK2. As shown in Fig. 7, HNF3α knockdown not only suppressed PSA and KLK2 expression but also significantly enhanced the effect of MSA on the expression of these two genes. MSA treatment induced an 80-90% reduction of PSA and KLK2 level in the HNF3α-knockdown cells, as opposed to ~50% reduction in the scrambled control siRNA-transfected cells. We next examined the impact of

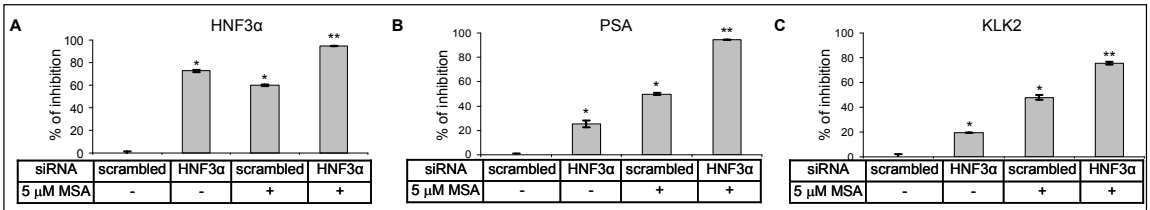

Fig. 7. Enhanced MSA (5 μM) inhibition of PSA and KLK2 by HNF3α knockdown in LNCaP cells as detected by real-time RT-PCR. The results are presented as % of inhibition compared to untreated scrambled control. *, statistically different (*P* < 0.05) compared to the untreated scrambled control. **, statistically different (*P* < 0.05) compared to the untreated and MSA-treated scrambled samples.

HNF3α knockdown on MSA-mediated growth inhibition by BrdU ELISA assay. As shown in Fig. 8, with the treatment of MSA, DNA synthesis inhibition rose from ~50% in the scrambled control cells to almost 100% in the HNF3α-knockdown cells. In addition, HNF3α knockdown was able to markedly inhibit DNA synthesis in the absence of MSA, indicating an important role of HNF3α in regulating cell proliferation in prostate cancer cells. The knockdown experiments were performed with 5 μM MSA (instead of 10 μM) in order to leave room to detect an enhanced effect of MSA by HNF3α knockdown. The impact of low HNF3α abundance as a result of MSA treatment on AR signaling and cell growth is currently being further investigated.

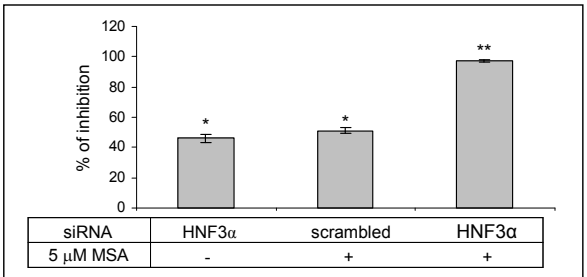

Fig. 8. Enhanced MSA (5 μM) inhibition of DNA synthesis by HNF3α knockdown in LNCaP cells as detected by ELISA of BrdU incorporation. The results are presented as % of inhibition of BrdU incorporation compared to untreated scrambled control. *, statistically different (*P* < 0.05) compared to the untreated scrambled control. **, statistically different (*P* < 0.05) compared to the untreated and MSA-treated scrambled samples.

C. KEY RESEARCH ACCOMPLISHMENTS:

- Selenium upregulates GKLF mRNA level through increasing GKLF transcription.
- Overexpression of GKLF enhances selenium inhibition of DNA synthesis in the AR-null PC-3 cells.
- Overexpression of GKLF induces apoptosis and enhances the effect of selenium on apoptosis induction in PC-3 cells.
- GKLF knockdown weakens the DNA synthesis suppressive activity of selenium in PC-3 cells.
- GKLF knockdown attenuates the apoptosis induction activity of selenium in PC-3 cells.
- Selenium treatment significantly decreases the expression of androgen receptor (AR) and prostate specific antigen (PSA) in five human prostate cancer cell lines (LNCaP, LAPC-4, CWR22Rv1, LNCaP-C81 and LNCaP-LN3), irrespective of their AR genotype (wild-type *vs.* mutant) or sensitivity to androgen-stimulated growth.
- Selenium inhibition of five AR-regulated genes implicated in prostate carcinogenesis (PSA, KLK2, ABCC4, DHCR24, and GUCY1A3) is significantly attenuated by AR overexpression.
- Transfection of AR in LNCaP cells weakens significantly the inhibitory effect of selenium on cell growth and proliferation.

- Selenium downregulates AR mRNA level through inhibiting AR transcription initiation.
- The *cis* element(s) mediating selenium downregulation of AR is present in a 1.7 kb proximal promoter region (-600 to +1115).
- Selenium treatment does not alter the ligand-binding capacity or affinity of AR.
- Selenium treatment leads to a significant suppression of AR N-C dimerization.
- Selenium treatment downregulates the expression level and DNA-binding activity of an AR-coregulator, HNF3α, leading to a decreased *trans*-activating activity of AR.

D. REPORTABLE OUTCOMES:

- ***Publications:***

  Yan Dong, Haitao Zhang, Allen C. Gao, James R. Marshall, and Clement Ip. (2005) Androgen receptor signaling intensity is a key factor in determining the sensitivity of prostate cancer cells to selenium inhibition of growth and cancer-specific biomarkers. *Molecular Cancer Therapeutics, 4, 1047-1055.*

  Soo O. Lee, Jae Y. Chun, Nagalakshmi Nadiminty, Donald L. Trump, Clement Ip, Yan Dong, and Allen C. Gao. (2006) Monomethylated selenium inhibits growth of LNCaP human prostate cancer xenograft accompanied by a decrease in the expression of androgen receptor and prostate-specific antigen (PSA). *Prostate,* 66, 1070-1075.

  Haitao Zhang, Yue Wu, Barbara Malewicz, Junxuan Lu, Song Li, James Marshall, Clement Ip, and Yan Dong. (2006) Augmented Suppression of Androgen Receptor Signaling by a Combination of α-Tocopheryl Succinate and Methylseleninic Acid. *Cancer, 107, 2942-2948.*

  Shuang Liu, Haitao Zhang, Liyu Zhu, Lijuan Zhao, and **Yan Dong.** (2008) KLF4 is a novel mediator of selenium in growth inhibition. *Molecular Cancer Research,* 6, 306–13.

- ***Abstract:***

  8th International Symposium on Selenium in Biology and Medicine, July, 2006, Madison, Wisconsin "Molecular Mechanisms Underlying Selenium Suppression of Androgen Receptor Signaling in Prostate Cancer Cells".

  2007 AACR annual Conference, April 14-18, 2007, Los Angeles, CA "Molecular Mechanisms Underlying Selenium Suppression of Androgen Receptor Signaling in Prostate Cancer Cells".

  1st International Symposium on Prostate Cancer, July 20-23, 2007, Changchun, Jilin, P.R. China "Androgen-Signaling Suppression in Mediating the Anticancer Effect of Selenium in Prostate Cancer".

- ***Presentations:***

  Roswell Park Cancer Institute Chemotherapy/Chemoprevention Research Round, January, 2006, Oral Presentation, "Androgen-Signaling Suppression in Selenium Chemoprevention of Prostate Cancer".

Jilin University, Changchun, P.R. China, June, 2006, Oral Presentation, "Selenium Chemoprevention of Prostate Cancer".

8th International Symposium on Selenium in Biology and Medicine, July, 2006, Poster Presentation, "Molecular Mechanisms Underlying Selenium Suppression of Androgen Receptor Signaling in Prostate Cancer Cells".

2007 AACR annual Conference, April 14-18, 2007, Poster Presentation, "Molecular Mechanisms Underlying Selenium Suppression of Androgen Receptor Signaling in Prostate Cancer Cells".

1st International Symposium on Prostate Cancer, July 20-23, 2007, Oral Presentation, "Androgen-Signaling Suppression in Mediating the Anticancer Effect of Selenium in Prostate Cancer".

Tulane University, New Orleans, LA, May, 2007, Oral Presentation, "Androgen-Signaling Suppression in Mediating the Anticancer Effect of Selenium in Prostate Cancer".

- ***Funding received:***

NCI Howard Temin (K01) Career Development Award (Dong, PI) 04/05 – 03/10

- Award per year: $120,000

Jilin Provincial Scholarship for Outstanding Scientists, Jilin Province, P.R. China (Dong, PI) 02/07 – 01/09

- Total direct cost: US $33,333

American Cancer Society Research Scholar Grant (Dong, PI) 7/1/07 – 6/30/11

- Award per year: $150,000

- ***Employment received:***

Assistant Professor, Dept. of Structural and Cellular Biology, Tulane University School of Medicine, New Orleans, LA

Program Member, Louisiana Cancer Research Consortium, New Orleans, LA

Assistant Member, Dept. of Cancer Chemoprevention, Roswell Park Cancer Institute, Buffalo, NY

Member of Graduate Faculty, Graduate Program of Cancer Pathology and Prevention, Roswell Division of Graduate School, State University of New York at Buffalo

Adjunct Professor, Dept. of Pathophysiology, School of Basic Medicine, Jilin University, Changchun, Jilin, P.R. China

E. CONCLUSIONS:

The results from the current study indicate an important role of GKLF induction in mediating the growth suppressive and apoptosis induction activities of selenium in the AR-null PC-3 cells. However, in cells expressing a functional AR, the disruption of AR signaling is most likely more important than the induction of GKLF signaling for selenium action. The vast

majority of prostate cancers express a functional AR. Although GKLF has growth suppressive activity in the AR-null cells, such activity might be overshadowed by AR signaling in AR-expressing cells. Therefore, it would be imperative to continue our research effort on the study of selenium suppression of AR signaling.

Almost all patients with advanced prostate cancer respond initially to treatments that interfere with the AR signaling process. However, these treatments often fail after prolonged use and recurrence becomes a major clinical issue (7). The development of hormone refractory prostate cancer is not associated with loss of AR (8;9). Instead, the accumulation of several molecular alterations frequently leads to a lower threshold requirement of androgens for the proliferation and survival of prostate cancer cells. Amplification and/or overexpression of AR can hyper-sensitize cells to sub-physiological levels of androgens (10-13). A recent report by Chen *et al.* (10) claimed that increased AR expression is both necessary and sufficient to convert prostate cancer growth from androgen-dependent to -independent, and that AR antagonists may display agonistic activity in cells with elevated AR expression. In addition, AR gene mutations could result in a promiscuous receptor with a broad ligand-binding and *trans*-activation spectrum (14). A selenium intervention strategy aimed at diminishing the expression of AR could be helpful not only for reducing prostate cancer incidence, but also for preventing relapses after endocrine therapy. In addition, the fact that selenium suppresses AR signaling provides a sound rationale for using selenium in combination with an anti-androgen as a new modality for not only the prevention but also the treatment of prostate cancer.

F. List of personnel receiving pay from the research effort

Yan Dong, PhD, PI

Dorothy Donovan, Laboratory Technician

Song Li, Predoctoral Student

Haitao Zhang, Chemoprevention Scientist

Shuang Liu, Postdoctoral Fellow

Bo Cao, Predoctoral Student

G. REFERENCES:

(1) Brinkmann AO, Blok LJ, de Ruiter PE, Doesburg P, Steketee K, Berrevoets CA, et al. Mechanisms of androgen receptor activation and function. J Steroid Biochem Mol Biol 1999 Apr;69(1-6):307-13.

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Androgen receptor signaling intensity is a key factor in determining the sensitivity of prostate cancer cells to selenium inhibition of growth and cancer-specific biomarkers

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Abstract

Our previous report showed that methylseleninic acid (MSA) significantly decreases the expression of androgen receptor and prostate-specific antigen (PSA) in LNCaP cells. The present study extended the above observations by showing the universality of this phenomenon and that the inhibitory effect of MSA on prostate cancer cell growth and cancer-specific biomarkers is mediated through androgen receptor down-regulation. First, MSA decreases the expression of androgen receptor and PSA in five human prostate cancer cell lines (LNCaP, LAPC-4, CWR22Rv1, LNCaP-C81, and LNCaP-LN3), irrespective of their androgen receptor genotype (wild type versus mutant) or sensitivity to androgen-stimulated growth. Second, by using the ARE-luciferase reporter gene assay, we found that MSA suppression of androgen receptor transactivation is accounted for primarily by the reduction of androgen receptor protein level. Third, MSA inhibition of five androgen receptor–regulated genes implicated in prostate carcinogenesis (*PSA*, *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*) is significantly attenuated by androgen receptor overexpression. Fourth, transfection of androgen receptor in LNCaP cells weakened noticeably the inhibitory effect of MSA on cell growth and proliferation. Androgen receptor signaling has been documented extensively to play an important role in the development of both androgen-dependent and -independent prostate cancer. Our finding that MSA reduces androgen receptor availability by blocking androgen receptor transcription provides justification for a mechanism-driven intervention strategy in using selenium to control prostate cancer progression. [Mol Cancer Ther 2005;4(7):1047–55]

Introduction

Prostate cancer is the second most common cancer and the second leading cause of cancer death in men in the United States. Androgen plays an important role not only in maintaining the function of the prostate but also in promoting the development of prostate cancer (1). Androgen binds to the androgen receptor, which subsequently translocates to the nucleus and interacts with specific androgen-responsive elements (ARE) on the promoters of target genes. The interaction leads to the activation or repression of genes involved in the proliferation and differentiation of the prostate cells (2). Prostate-specific antigen (PSA) and kallikrein 2 (KLK2) are two well-accepted targets of androgen receptor. PSA, also known as kallikrein 3, is an established serum marker for the diagnosis and prognosis of prostate cancer. Although KLK2 is not as widely used as PSA, it is increasingly recognized to provide added information to disease staging (3, 4).

The randomized, placebo-controlled Nutritional Prevention of Cancer trial showed that selenium supplementation reduced the incidence of prostate cancer by 50% (5, 6). This trial was designed initially to assess the effect of selenium on nonmelanoma skin cancer. Because men accounted for a sizable proportion of the cohort (974 of a total of 1,312), there was sufficient power to analyze the changes in prostate cancer risk. When the prostate cancer data were further stratified, there was evidence of a greater reduction in risk from selenium supplementation among men who had low baseline plasma PSA levels (6). Early-stage prostate cancer is mostly responsive to androgen stimulation. The inference that the protective effect of selenium might be more pronounced in early-stage prostate cancer, as reflected by low PSA secretion, lends credence to the idea that selenium might affect androgen signaling.

Recently, we reported that a selenium metabolite, in the form of methylseleninic acid (MSA), greatly down-regulates the expression of androgen receptor and PSA in the androgen-responsive LNCaP human prostate cancer cells (7, 8). The suppression of androgen receptor signaling occurs well before any significant growth inhibition, which is accompanied by correlative changes in numerous cell

Received 4/25/05; accepted 5/11/05.

Grant support: Department of Defense Postdoctoral Fellowship Award and New Investigator Award W81XWH-04-1-0009 (Y. Dong); Roswell Park Alliance Foundation (C. Ip); National Cancer Institute grant CA91990 (C. Ip); and National Cancer Institute, Cancer Center Support Grant P30 CA16056 (Roswell Park Cancer Institute).

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cycle and apoptosis regulatory molecules (9–13). Androgen receptor signaling involves multiple steps, the receptor itself is just one of many effectors that participate in the process. For example, heat shock proteins are known to modulate the stability of androgen receptor as well as its affinity to androgen (14, 15). The transactivating activity of androgen receptor can be affected markedly by a large number of coactivators and corepressors (16). Our microarray analysis suggests that MSA alters the expression of several heat shock proteins, coactivators, and corepressors of the superfamily of steroid hormone receptors (17). In view of these confounding effects, the present study was designed to determine the role of androgen receptor down-regulation per se in MSA interference of androgen receptor signaling. Our approach was to use the ARE-luciferase reporter gene assay to find out the extent to which selenium suppression of androgen receptor transactivation could be reversed when the luciferase activity is normalized based on androgen receptor protein level. We also investigated whether androgen receptor transfection might attenuate selenium-mediated down-regulation of five androgen receptor targets: *PSA*, *KLK2*, ATP-binding cassette C4 (*ABCC4*, also known as *MRP4*), 24-dehydrocholesterol reductase (*DHCR24*, also known as *seladin-1*), and soluble guanylate cyclase 1 α 3 (*GUCY1A3*). These five androgen-inducible genes were selected based on the criteria that they are consistently overexpressed in prostate cancer compared with normal prostate tissue (18). Finally, in an effort to evaluate the biological significance of the selenium-androgen receptor signaling axis, we investigated whether androgen receptor overexpression might block the growth inhibitory effect of selenium.

Materials and Methods

Selenium Reagent, Prostate Cancer Cell Lines, 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay, and Bromodeoxyuridine-Labeling Analysis

MSA was synthesized as previously described (19). The LNCaP and CWR22Rv1 human prostate cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). The LAPC-4 cell line was provided by Dr. Charles L. Sawyers at the University of California at Los Angeles Jonsson Comprehensive Cancer Center. The two androgen-unresponsive LNCaP sublines, LNCaP-LN3 and LNCaP-C81, were obtained from Dr. Curtis A. Pettaway (University of Texas M.D. Anderson Cancer Center) and Dr. Ming-Fong Lin (University of Nebraska Medical Center), respectively. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell growth and the bromodeoxyuridine (BrdUrd) labeling for DNA synthesis were done as described in our previous publication (17).

Transient Transfection of Androgen Receptor

The procedure was carried out using the Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA) per instruction of the manufacturer. At 24 hours before transfection, cells were plated in growth medium without antibiotics at a density to reach 90% to 95% confluency at transfection. The pSG5hAR androgen receptor expression vector (20) or the pSG5 mock plasmid (Stratagene, La Jolla, CA) was introduced into LNCaP cells with or without the cotransfection of the pEGFP-F membrane-GFP-encoding construct (BD Biosciences, San Jose, CA). The purpose of the green fluorescent protein (GFP) was to enable us to enrich for the subset of positively androgen receptor–transfected cells. During cotransfection, the two plasmids were added at 1:1 molar ratio. The amount of DNA transfected was 12 μg per 10-cm culture dish. The DNA/liposome mixture was removed at 3 hours after transfection. For the MTT assay, the cells were trypsinized 16 hours later and plated in triplicate onto a 96-well plate. Cells were allowed to recover for an additional 24 hours before exposure to 10 μmol/L MSA. The MTT assay was conducted at 48 hours post-MSA treatment. For the BrdUrd-labeling analysis, the cells were subjected to MSA treatment at 24 hours posttransfection and labeled with BrdUrd after 24 hours of MSA treatment.

Reporter Gene Assay

The ARE-luciferase reporter plasmid, containing three repeats of the ARE region ligated in tandem to the luciferase reporter (20), was transiently transfected into cells at a concentration of 9 μg per 10-cm culture dish. After incubating with the transfection mixture for 3 hours, the cells were trypsinized, resuspended in medium containing charcoal-stripped serum and 10 nmol/L dihydrotestosterone (Sigma, St. Louis, MO), and plated in triplicate onto 6-well plates. Cells were allowed to recover for an additional 24 hours before exposure to 10 μmol/L MSA. After 6 or 16 hours of MSA treatment, cells were lysed in reporter lysis buffer (Promega, Madison WI), and the luciferase activity was assayed using the Luciferase Assay System (Promega). Protein concentration in cell extracts was determined by the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Luciferase activities were normalized by the protein concentration of the sample. The transfection experiments were repeated thrice.

Western Blot Analysis

Details of the procedure for Western blot analysis were described previously (17). Immunoreactive bands were quantitated by volume densitometry and normalized to glyceraldehyde-3-phosphate dehydrogenase. The following monoclonal antibodies were used in this study (source): anti-glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Temecula, CA), anti–androgen receptor (BD Biosciences), and anti-PSA (Lab Vision, Fremont, CA).

Real-time Reverse Transcription-PCR

Real-time reverse transcription-PCR analysis was done as described previously (21). The PCR primers and

Taqman probes for β-actin, androgen receptor, PSA, KLK2, ABCC4, DHCR24, and GUCY1A3 were Assays-on-Demand products from Applied Biosystems (Foster City, CA). The PCR conditions were as follows: an initial incubation at 50°C for 2 minutes, then a denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative quantitation of gene expression was done using the comparative C_T ($\Delta\Delta C_T$) method (22).

Androgen Receptor mRNA Stability Assay

Actinomycin D (5 μg/mL) was added to the cultures to stop new RNA synthesis at the time of MSA treatment, and androgen receptor mRNA levels were measured by real-time reverse transcription-PCR at hourly intervals for the next 6 hours. The turnover of androgen receptor mRNA was determined by comparing mRNA levels over time in cells treated with or without MSA.

Statistical Analysis

The Student's two-tailed *t* test was used to determine significant differences between treatment and control values, and $P < 0.05$ was considered statistically significant.

Results

MSA Depresses Androgen Receptor Transcription

Figure 1 shows the effect of MSA on androgen receptor transcript and protein levels as well as androgen receptor mRNA stability in LNCaP cells. The decrease in androgen receptor transcript, as determined by real-time reverse transcription-PCR, occurred very quickly (Fig. 1A). On the average, there was about a 50% reduction in the first three hours after treatment with 10 μmol/L MSA; by 6 hours, the magnitude of inhibition rose to 80%. At the protein level, there was no change in androgen receptor in the first two hours (Fig. 1B). A modest decrease began to appear at 3 hours, and the inhibition became very pronounced at 6 hours (Fig. 1B). The observation is consistent with the time-dependent sequence of reduced mRNA leading to decreased protein expression. To determine whether the down-regulation of androgen receptor mRNA was due to decreased transcription or increased mRNA degradation, we did an mRNA stability assay under the condition in which new RNA synthesis was blocked. Actinomycin D was added to the culture at the time of MSA treatment, and androgen receptor mRNA levels were followed in a 6-hour time course experiment. Because actinomycin D could be cytotoxic, we also monitored cell growth for up to 8 hours and did not observe cell death or significant growth inhibition during this period. Our results showed that treatment with MSA actually increased the stability of androgen receptor mRNA (Fig. 1C). This observation rules out increased mRNA degradation as a contributing factor. Therefore, the decrease in androgen receptor mRNA level by MSA is likely to be accounted for by a vigorous block of androgen receptor transcription.

We next examined the effect of MSA on the expression of androgen receptor and PSA in four additional human prostate cancer cell lines: LAPC-4, CWR22Rv1, LNCaP-C81, and LNCaP-LN3. The LAPC-4 cells are androgen responsive and express a wild-type androgen receptor (23), as opposed to LNCaP cells that are also androgen responsive but express a mutant, although functional, androgen receptor. The other three cell lines are all androgen-unresponsive and express a mutant but functional androgen receptor (24–27). As shown in Fig. 2 (*left*), MSA decreased androgen receptor and PSA transcript levels progressively as a function of time in all four cell lines examined. The reduction in androgen receptor and PSA proteins (*right*) paralleled the drop in the transcripts. In LAPC-4, CWR22Rv1, and LNCaP-C81 cells, a decrease in PSA transcript was already detectable as early as 3 hours, at a time when there was no apparent loss of the androgen receptor protein. The data suggest that MSA disrupts androgen receptor signaling through additional mechanism(s) beyond reducing the availability of the androgen receptor protein.

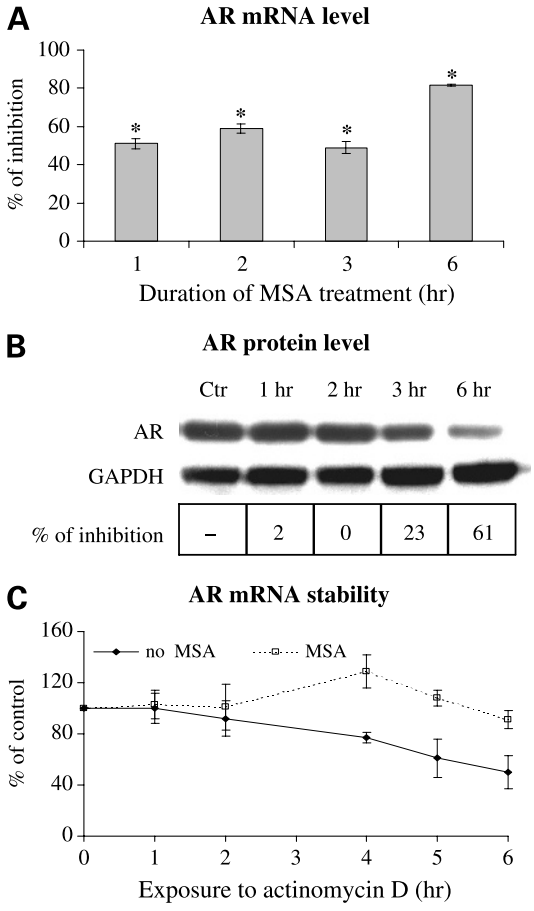

Figure 1. Effect of MSA on androgen receptor (*AR*) expression in LNCaP cells. **A,** inhibition of androgen receptor mRNA level as determined by real-time RT-PCR. **B,** inhibition of androgen receptor protein level as determined by Western blot analysis. **C,** androgen receptor mRNA stability in the presence or absence of MSA. *Bars,* SE. *, $P < 0.05$, statistically different compared with untreated control.

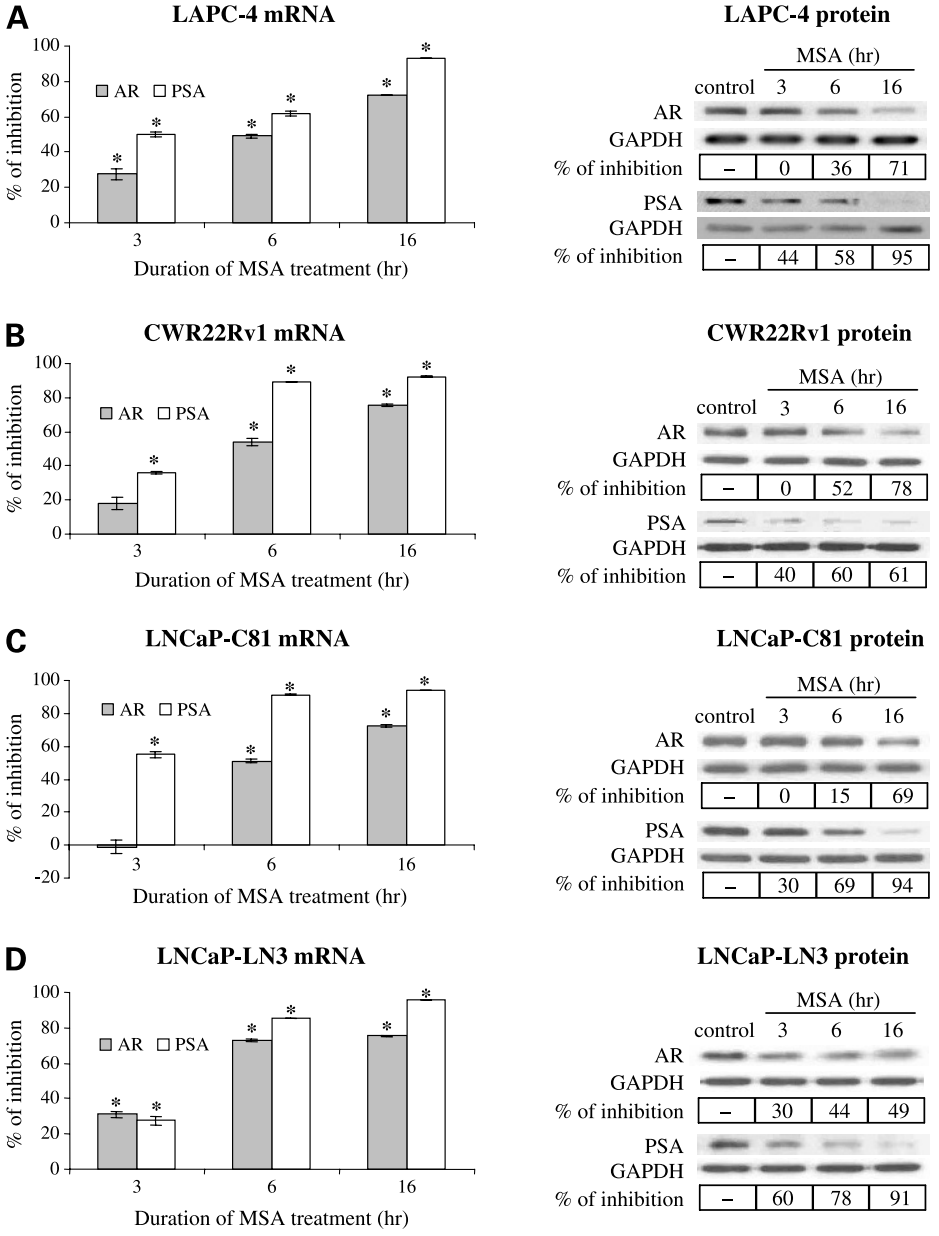

Figure 2. Effect of MSA on androgen receptor (*AR*) and PSA expression in LAPC-4, CWR22Rv1, LNCaP-C81, and LNCaP-LN3 cells. *Left,* mRNA levels as determined by real-time RT-PCR; *right,* protein levels as determined by Western blot analysis. *Columns,* % inhibition; *bars,* SE. *, $P < 0.05$, statistically different compared with untreated control.

MSA-Mediated Androgen Receptor Down-Regulation Leads to a Reduction of Androgen Receptor Transactivating Activity

The transactivation of androgen receptor is an indicator of androgen receptor signaling and can be quantified readily by a reporter gene assay. To investigate whether the reduced availability of androgen receptor by MSA is a major factor in modulating androgen receptor transcriptional activity, we transiently transfected LNCaP cells with the ARE-luciferase reporter plasmid and normalized the luciferase activity based on the level of the androgen receptor protein. This normalization step eliminates the level of androgen receptor expression as a determinant of androgen receptor transactivation. The luciferase reporter assay was carried out at 6 and 16 hours after treatment with 10 μmol/L MSA. At these two time points, androgen receptor protein level was inhibited by 60% and 77%, respectively (Fig. 3B, *inset*). As can be seen in Fig. 3A, without normalizing for the difference in androgen receptor protein level between the MSA-treated and -untreated samples, the ARE-promoter activity was decreased by 65% or 75%, respectively, after 6 or 16 hours of MSA treatment. However, after normalization, the ARE-promoter activity was inhibited by a meager 15% at the 6-hour time point, and the inhibition disappeared completely at 16 hours (Fig. 3B). These findings suggest that the reduced availability of the androgen receptor protein is the major factor in contributing to MSA disruption of androgen receptor signaling.

Overexpression of Androgen Receptor Attenuates the Effect of MSA on the Down-Regulation of Androgen Receptor–Regulated Genes

To delineate the role of low androgen receptor abundance as a cause of reduced PSA expression by selenium, we transiently transfected LNCaP cells with a wild-type androgen receptor construct and determined the response of PSA to MSA. After 3 hours of MSA exposure, PSA transcript was depressed by about 75% in the mock-transfected cells but only by about 45% in the androgen receptor–transfected cells (Fig. 4A). Based on our routine experience of a 40% transfection efficiency as determined by GFP cotransfection analysis (described below), we believe that the inhibitory effect of MSA on PSA mRNA might have been reversed completely in positive androgen receptor transfectants. Our conclusion was derived from the following theoretical calculation: 40% of $(1 - x)$ + 60% of $(1 - a) = 1 - b$, where x = % inhibition in positive androgen receptor transfectants, a = 75% inhibition in mock transfectants, and b = 45% inhibition in the mixed population of androgen receptor–transfected cells. Solving for x in the above equation gave a value of 0% inhibition. In other words, there was no inhibition of PSA expression by MSA in the positive androgen receptor transfectants (i.e., complete reversal). The difference between the mock- and androgen receptor–transfected cells, although still apparent, was not as great at 4 and 6 hours compared with that at 3 hours. The fact that a robust androgen receptor presence was not sufficient to completely counteract the suppressive effect of MSA on the transcription of PSA at the later time points suggests that there could be a delay in the recruitment of additional mechanisms by which MSA might diminish androgen receptor signaling. We also studied the protein level of PSA by Western blotting. The Western analysis was done at 24 hours after MSA treatment. As shown in Fig. 4B, PSA protein was depressed by about 70% in the mock-transfected cells but only by about 40% in the androgen receptor–transfected cells. The protein levels of androgen receptor in the mock- and androgen receptor–transfected cells are also shown in Fig. 4B for confirmation purposes.

Using a bioinformatic data mining approach, we recently identified five additional androgen-inducible genes that are expressed at a higher level in prostate cancer compared

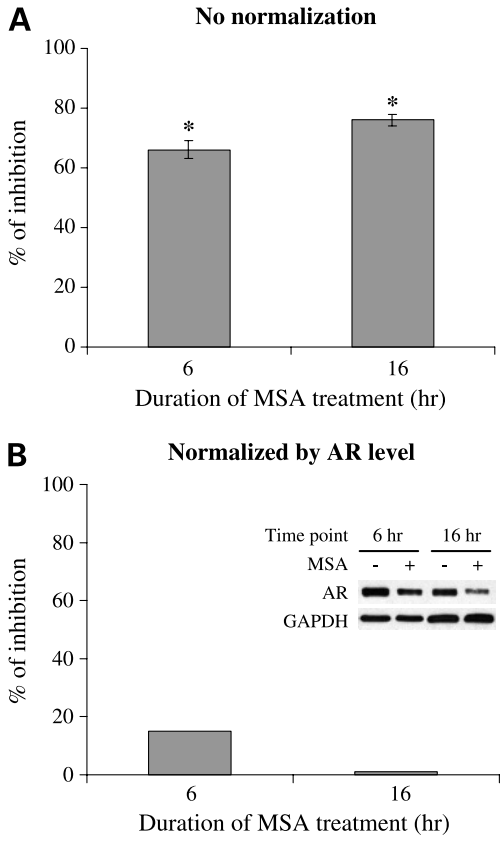

Figure 3. Effect of MSA on ARE-promoter activity before (**A**) and after (**B**) normalizing by androgen receptor (*AR*) protein level. **B,** representative Western blot analysis of androgen receptor protein level in the cell extracts (*inset*). Androgen receptor protein level was depressed by 60% or 77% at 6 or 16 h, respectively. *Columns,* % inhibition; *bars,* SE. *, $P < 0.05$, statistically different compared with untreated control.

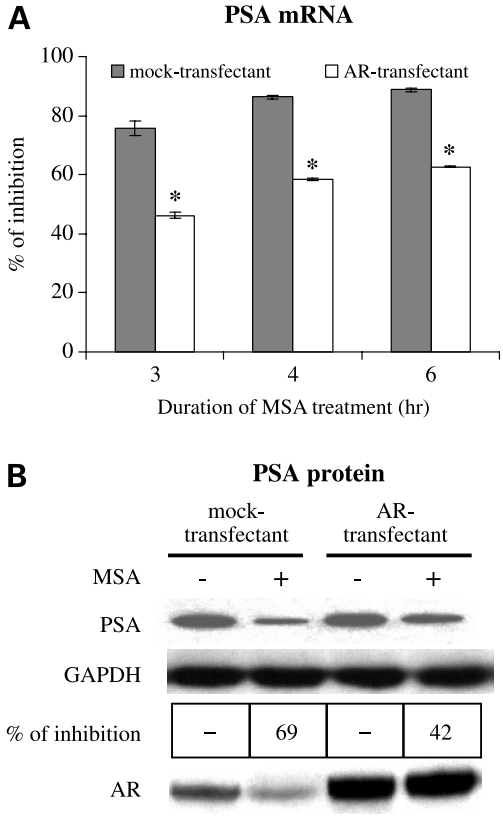

Figure 4. Effect of androgen receptor (*AR*) overexpression on MSA down-regulation of PSA. **A,** PSA mRNA expression as determined by real-time RT-PCR, in androgen receptor– or mock-transfected LNCaP cells treated with MSA. *Columns,* % inhibition. *, $P < 0.05$, statistically different compared with the value from the mock transfectant. **B,** PSA Western blot analysis in androgen receptor– or mock-transfected LNCaP cells treated with MSA.

with normal prostate tissue; furthermore, their expression is repressed by MSA (18). These genes are *KLK2*, *ABCC4* (also known as *MRP4*), *DHCR24* (also known as *seladin-1*), *GUCY1A3*, and long-chain fatty acid CoA ligase 3 (*FACL3*). MSA down-regulation of their expression only occurs in LNCaP cells but not in the androgen-unresponsive PC-3 cells that express an extremely low level of androgen receptor (18). To verify that the decreased expression of these genes is a direct consequence of MSA suppression of androgen receptor signaling, we applied the same androgen receptor overexpression protocol as described above and used real-time reverse transcription-PCR to quantitate their transcript levels. The *FACL3* gene was not included in this study as no Assays-on-Demand primers and probes are available for this gene. The results are shown in Fig. 5. Androgen receptor transfection significantly muted the inhibition of gene expression by MSA. In general, the difference in % inhibition between the mock and androgen receptor transfectants was greatest at 3 hours and narrowed gradually with time. The overall pattern was very similar for *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*. The data thus show a key role of androgen receptor down-regulation in mediating the inhibitory effects of MSA on the expression of androgen receptor–regulated genes.

Overexpression of Androgen Receptor Interferes with MSA-Mediated Growth Inhibition

In an effort to evaluate the biological significance of MSA suppression of androgen receptor signaling, we transiently transfected LNCaP cells with a wild-type androgen receptor and assessed the response of the androgen receptor–overexpressing cells to MSA-induced growth inhibition. The MTT assay was conducted at 48 hours post-MSA, and the data are presented in Fig. 6A. In the absence of MSA, cell growth was not altered by the transfection of androgen receptor (data not shown), indicating that the endogenous level of androgen receptor is not a limiting factor for the growth of these cells. MSA treatment inhibited growth by 40% in the mock transfectants, as opposed to 27% in the androgen receptor transfectants. The difference is statistically significant ($P = 0.003$). Thus, androgen receptor overexpression was able to weaken the growth suppressive activity of MSA. One reason that the difference was seemingly compressed was due to the fact that only a fraction of cells was successfully transfected, and in this study, cell growth was assessed using the whole cell population. To address the last problem, we cotransfected cells with the androgen receptor expression vector and a membrane-GFP-encoding construct. The cells were then subjected to BrdUrd labeling, and the data were analyzed by gating just the GFP-positive cells. As shown in Fig. 6B, after selecting for the subset of GFP-positive cells, we found that MSA inhibited DNA synthesis by a very modest 16% in the androgen receptor transfectants, as opposed to 72% in the mock transfectants. Because the GFP and androgen receptor cDNAs are not located in the same plasmid construct, it is possible that not all the cells positive for GFP are also positive for the transfected androgen receptor. Thus, our selection process only led to an enrichment, rather than an exclusive selection, of double-positive cells. Therefore, the difference

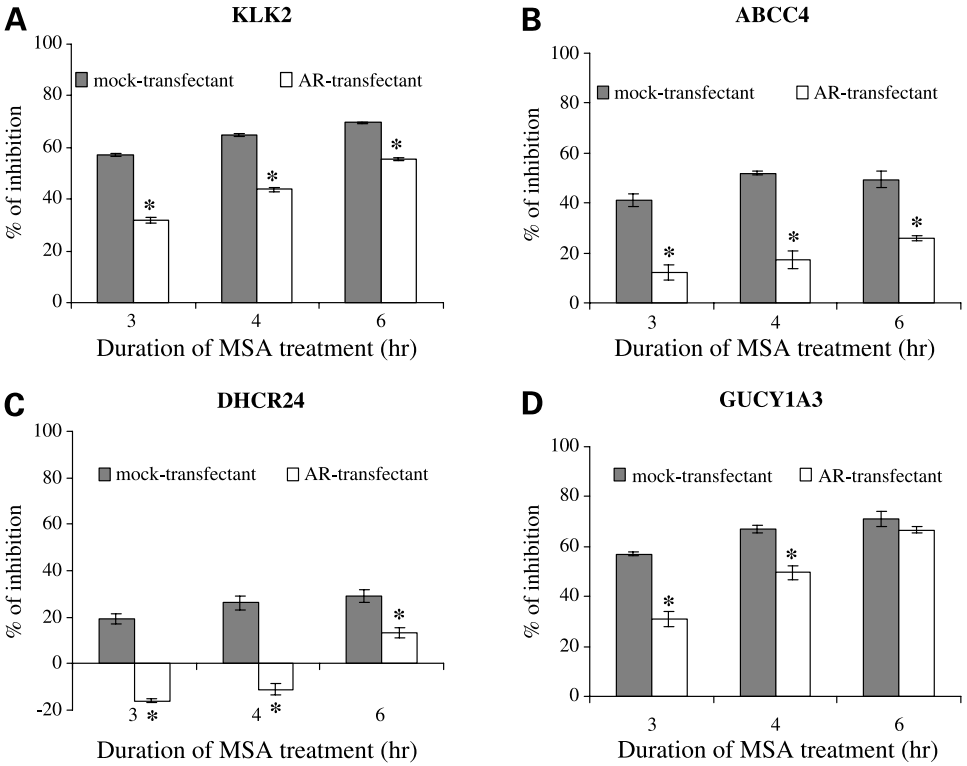

Figure 5. Effect of androgen receptor (*AR*) overexpression on MSA down-regulation of KLK2, ABCC4, DHCR24, and GUCY1A3 mRNA expression as determined by real-time RT-PCR, in androgen receptor– or mock-transfected LNCaP cells treated with MSA. *Columns,* % inhibition. *, $P < 0.05$, statistically different compared with the value from the mock transfectant.

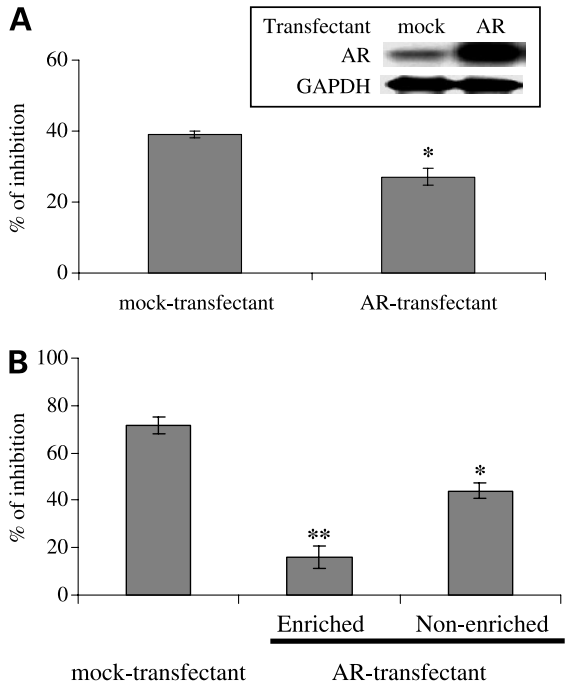

Figure 6. Effect of androgen receptor (*AR*) overexpression on MSA inhibition of cell growth. **A,** MTT cell growth assay in androgen receptor– or mock-transfected LNCaP cells treated with MSA. Western blot confirmation of androgen receptor protein level (*inset*). **B,** BrdUrd labeling of selected GFP-positive or nonselected androgen receptor–transfected LNCaP cells treated with MSA. *Columns,* % inhibition compared with untreated control. *, $P < 0.05$, statistically different from mock transfectant. **, $P < 0.05$, statistically different from mock transfectant and nonselected androgen receptor transfectant.

between the mock transfectants and the androgen receptor transfectants might have been even more pronounced if all the cells used in the experiment were successfully transfected with androgen receptor. Figure 6B also shows that when we did the BrdUrd labeling experiment with the nonenriched androgen receptor–transfected cells, the inhibition by MSA was about 45%, a value half-way between that achieved by the mock transfectants and the enriched androgen receptor transfectants.

Discussion

Our previous report showed that selenium significantly decreases the expression and the transactivating activity of androgen receptor in LNCaP cells (21). The present study extended the above observations by showing the universality of this phenomenon and a key role of androgen receptor down-regulation in mediating the inhibitory effects of selenium on prostate cancer cell growth and the expression of cancer-specific biomarkers. First, selenium decreases the expression of androgen receptor and PSA in five human prostate cancer cell lines, irrespective of their androgen receptor genotype (wild type versus mutant) or sensitivity to androgen-stimulated growth. Second, a reporter gene assay with the ARE-luciferase construct indicated that depletion of the androgen receptor protein is a major factor for selenium depression of androgen receptor transactivating activity. Third, overexpression of androgen receptor greatly weakens the inhibitory effects of selenium on prostate cancer cell proliferation as well as the expression of five androgen receptor–regulated genes implicated in prostate carcinogenesis: *PSA*, *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*. These findings, however, do not necessarily exclude additional mechanisms by which selenium diminishes androgen receptor signaling (e.g., via modulation of ligand binding, androgen receptor dimerization, nuclear translocation, and the interaction of androgen receptor with its coregulators). In fact, our previous report provided some evidence that selenium is able to inhibit the binding of androgen receptor to the ARE in the absence of a drop in the androgen receptor level (21).

A selenium intervention strategy aimed at diminishing the expression of androgen receptor could be helpful not only for reducing prostate cancer incidence but also for preventing relapses after endocrine therapy. Almost all patients with advanced prostate cancer respond initially to treatments that interfere with the androgen receptor–signaling process. However, these treatments often fail after prolonged use and recurrence becomes a major clinical issue (28). The development of hormone refractory prostate cancer is not associated with loss of androgen receptor (29, 30). Instead, the appearance of several molecular alterations frequently leads to a lower threshold requirement of androgens for the proliferation and survival of prostate cancer cells. Androgen receptor gene mutations could result in a promiscuous receptor with a broad ligand-binding and transactivation spectrum (31). Amplification and/or overexpression of androgen receptor may hypersensitize cells to subphysiologic levels of androgens (32–35). A recent report by Chen et al. (35) claimed that increased androgen receptor expression is both necessary and sufficient to convert prostate cancer growth from androgen-dependent to -independent and that androgen receptor antagonists may display agonistic activity in cells with elevated androgen receptor expression. On the other hand, several studies showed that knocking down the expression of androgen receptor inhibits the growth of prostate cancer cells, both *in vitro* and *in vivo*, and induces apoptosis (36–39). Because selenium blocks the transcription of androgen receptor (see Fig. 1), this treatment modality may prove to be effective in prostate cancer intervention.

The down-regulation of androgen receptor targets by selenium has important clinical implication. We have studied *PSA*, *KLK2*, *ABCC4*, *DHCR24*, and *GUCY1A3*. All these genes are expressed at a higher level in prostate cancer compared with normal prostate tissue (18). PSA and KLK2 are prostate-specific differentiation markers. They belong to the serine protease family and are both secretory proteins. PSA is the most useful serum marker for the diagnosis and prognosis of prostate cancer. The combined use of PSA and KLK2 has been shown to improve the specificity of biochemical detection of prostate cancer (40–44) and the accuracy in predicting tumor grade and

stage (3, 4). ABCC4 (also known as MRP4) is a member of the multidrug resistance-associated protein family of transporters. Overexpression of ABCC4 in neuroblastoma is associated with poor prognosis and resistance to the topoisomerase I poison irinotecan and its active metabolite SN-38 (45). Thus, the down-regulation of MRP4 by selenium might represent a potential mechanism by which selenium enhances the therapeutic efficacy of a number of anticancer drugs, including irinotecan (46). DHCR24 (also known as seladin-1) is an antiapoptotic protein, it inhibits the activity of caspase 3 (47). The overexpression of this gene has also been reported in adrenocortical adenoma cells compared with adjacent nontumor cells (48). GUCY1A3 catalyzes the conversion of GTP to the second messenger cyclic guanosine 3′,5′-monophosphate, which regulates the activity of protein kinases, phosphodiesterases, and ion channels (49). Future selenium intervention trial may consider monitoring androgen receptor, PSA, KLK2, ABCC4, DHCR24, and GUCY1A3 in biopsied prostate samples, to obtain a more comprehensive picture of an individual's responsiveness to selenium. Recent data also showed that cellular PSA is more sensitive than secretory PSA to selenium intervention (50). This is one more reason why it is preferable to do the analysis in biopsied prostate tissue.

Acknowledgments

We thank Hai Wang, Dorothy Donovan, Tamora Loftus, Todd Parsons, and Cathy Russin for their excellent technical assistance.

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Krüppel-Like Factor 4 Is a Novel Mediator of Selenium in Growth Inhibition

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Abstract

A previous prevention trial showed that selenium supplementation was effective in reducing (by 50%) the incidence of prostate cancer. Selenium has been reported to inhibit the growth of prostate cancer cells *in vitro*. Multiple mechanisms are likely to be operative in the underlying effect of selenium. Here, we report that Krüppel-like factor 4 (KLF4), a transcription factor of the KLF family, is an important target of selenium. We found that selenium up-regulates KLF4 expression and increases the DNA-binding activity of KLF4 in both the androgen-dependent LNCaP and the androgen-independent PC-3 human prostate cancer cells. The increase of KLF4 mRNA is accounted for primarily by enhanced transcription, although the contribution of a slight abatement in mRNA degradation cannot be ruled out. KLF4 knockdown using short interference RNA significantly weakens the effects of selenium on DNA synthesis inhibition, apoptosis induction, and the expression of three KLF4 target genes, cyclin D1, p21/WAF1, and p27/Kip1. In addition, the overexpression of KLF4 not only leads to an induction of apoptosis in the control cells, but also enhances the DNA synthesis–suppressive and–proapoptotic activities of selenium. Taken together, our results suggest that KLF4 plays a key role in mediating the growth-inhibitory effect of selenium in prostate cancer cells.
(Mol Cancer Res 2008;6(2):306–13)

Introduction

KLF4, also known as gut-enriched Krüppel-like factor (GKLF) or epithelial zinc finger, is a member of the Krüppel-like factor (KLF) family of proteins (1). KLF proteins are known to play an important role in differentiation and development (2, 3). They are zinc finger transcription factors, characterized by a COOH-terminal DNA-binding domain which consists of three C_2H_2 zinc fingers (2, 3). KLF4 is highly expressed in terminally differentiated epithelial cells of the skin and gastrointestinal tract (4, 5). It regulates the expression of a number of genes essential for cell cycle progression (e.g., cyclin D1, cyclin B1, p21/WAF1, p27/Kip1, inhibitor of DNA-binding 3, ornithine decarboxylase 1), and is intimately involved in controlling G_1-S and G_2-M checkpoints following DNA damage (6-13). The level of KLF4 generally increases in response to serum deprivation (13), contact inhibition (1), and heat stress (14).

Depending on cell type or cell context, KLF4 may act either as a tumor suppressor gene or as an oncogene (15, 16). The function of KLF4 in carcinogenesis has been studied more extensively in gastrointestinal cancer than in other cancers. KLF4 is down-regulated in colon (17, 18), gastric (19), and esophageal (20-22) cancers. The reduction is due to either a loss of heterozygosity of the KLF4 locus, hypermethylation of the 5′-untranslated region, or point mutations in the coding region (18). Overexpression of KLF4 is known to decrease the tumorigenicity of colonic cancer cells (23). On the other hand, KLF4 knockout mice have been reported to manifest differentiation defects and precancerous changes in the stomach (24). Consistent with its tumor suppressor activity in gastrointestinal tumors, decreased KLF4 expression has also been observed in other types of cancer, including prostate (25, 26), bladder (27), lung (28), and T-cell leukemia (29). Conversely, the role of KLF4 as an oncogene is supported by the finding of an increased expression in oral dysplastic epithelium, squamous cell carcinoma (30), and breast cancer (25) when compared with the corresponding normal tissues. Given that the deregulation of KLF4 is a common occurrence in organ site carcinogenesis, identifying a corrective strategy and studying its consequence on cancer cell growth would be an endeavor worthy of pursuit.

Prostate cancer is characterized by a long latency period and is therefore most suited for testing the idea of intervention. In a previous randomized, placebo-controlled cancer prevention trial, supplementation with a nutritional dose of selenium was found to reduce prostate cancer incidence by 50% (31, 32). *In vitro* studies showed that selenium inhibits human prostate cancer cell growth, blocks cell cycle progression, and induces programmed cell death (33, 34). In an effort to delineate the molecular targets underlying the anticancer action of selenium, we did a microarray analysis to profile gene expression changes

Received 4/6/07; revised 10/2/07; accepted 10/9/07.
Grant support: National Cancer Institute grant no. K01 CA114252 (Y. Dong), DOD New Investigator grant nos. W81XWH-04-1-0009 (Y. Dong) and W81XWH-05-1-0598 (H. Zhang), Jilin Provincial Scholarship for Outstanding Scientists from Jilin Province, China (Y. Dong), and partially supported by shared resources of the Roswell Park Cancer Center Support grant P30 CA16056 from the National Cancer Institute.
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doi:10.1158/1541-7786.MCR-07-0159

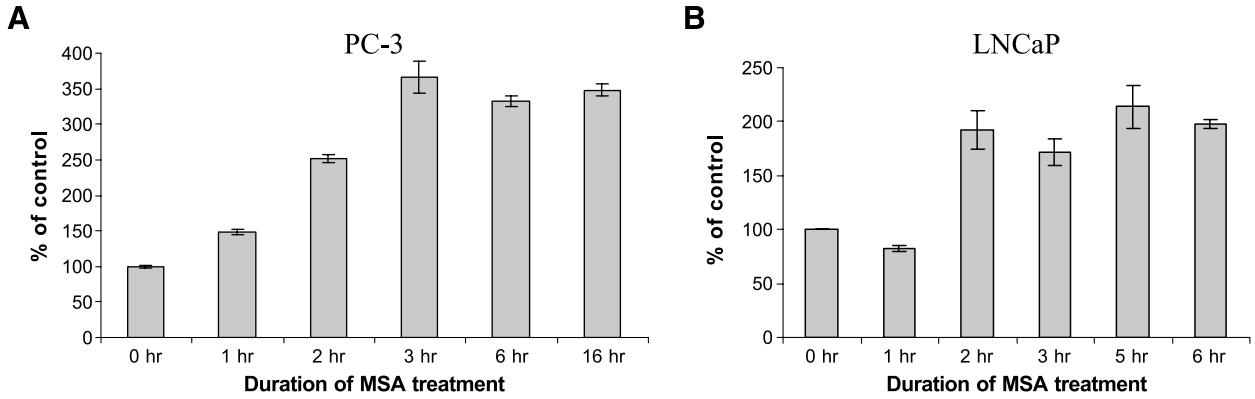

FIGURE 1. Effect of MSA on KLF4 mRNA expression as determined by real-time RT-PCR. Columns, mean percentage of control; bars, SE. With the exception of the 1-h data point in LNCaP cells, the remaining data points are statistically different ($P < 0.01$) from the untreated control.

mediated by selenium in the PC-3 human prostate cancer cells (33). KLF4 was identified as one of the early selenium-responsive genes. A rapid induction of KLF4, accompanied by the altered expression of several known KLF4 targets (e.g., cyclin D1, p21/WAF1, p27/Kip1), were observed in response to selenium treatment. Based on the above information, it would be reasonable to hypothesize that KLF4 is an important proximal mediator of the action of selenium. In this report, we followed up on our microarray data by examining the up-regulation of KLF4 and the modulation of KLF4 targets by selenium. Additionally, we evaluated how KLF4 knockdown or overexpression might modify the antiproliferative and proapoptotic activities of selenium. The selenium compound used in the study is methylseleninic acid (CH_3SeO_2H, abbreviated to MSA), which was developed specifically for *in vitro* experiments (35). Once taken up by cells, MSA is rapidly reduced to the active metabolite, methylselenol (which is rather unstable in itself), via a nonenzymatic reaction involving glutathione and NADPH.

Results

MSA Up-Regulates KLF4 mRNA Expression

We assessed KLF4 mRNA level by quantitative real-time reverse transcription-PCR (RT-PCR) in PC-3 and LNCaP human prostate cancer cells. Cells were treated with 10 μmol/L of MSA for various lengths of time as indicated in Fig. 1. In both cell lines, a ~2-fold induction of KLF4 mRNA was detected as early as 2 h after exposure to MSA. In LNCaP cells, the increase plateaued off at this point, whereas in PC-3 cells, it continued to increase to ~3.5-fold at 3 h, and remained at this level for at least up to 16 h. We next set out to examine the level of KLF4 protein in response to MSA treatment. Unfortunately, none of the commercially available KLF4 antibodies produced a specific signal on Western blots.

MSA Increases KLF4 mRNA Stability and Transcription Initiation

To determine whether the up-regulation of KLF4 mRNA was a result of decreased mRNA degradation or increased transcription, we carried out an mRNA stability assay under conditions in which new RNA synthesis was blocked. Actinomycin D was added to the culture at the time of MSA treatment, and KLF4 mRNA levels were followed in a 6-h time course experiment (Fig. 2). Our results showed that MSA treatment modestly increased the stability of KLF4 mRNA in PC-3 cells. The shift in the decay curve was less notable in LNCaP cells.

We then studied the effect of MSA on KLF4 transcription by nuclear run-on assay. Biotin-labeled nascent transcripts obtained by run-on transcription were isolated by using streptavidin particle beads, and quantitated by real-time RT-PCR. As shown in Fig. 3, MSA treatment resulted in a 2- to 3-fold induction of KLF4 transcription at the 3- and 6-h time points in both cell lines. The data thus indicate that the elevation of KLF4 mRNA level by MSA is primarily due to an

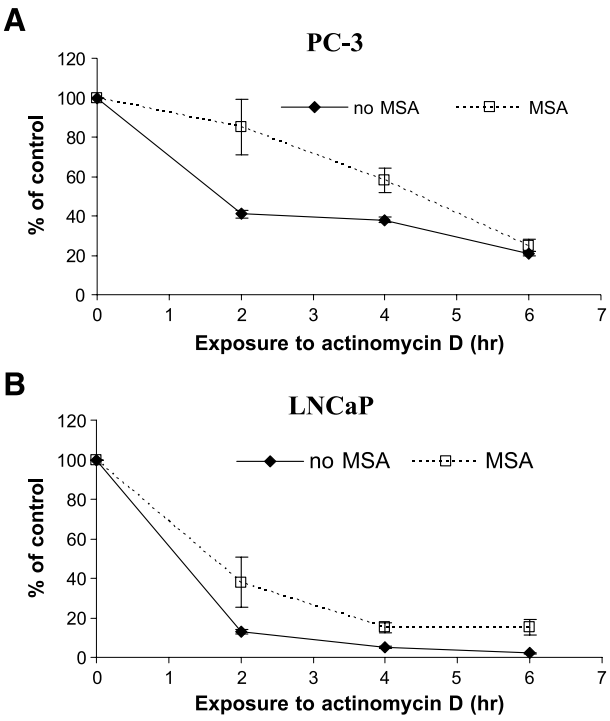

FIGURE 2. Effect of MSA on KLF4 mRNA stability. Points, mean percentage of control; bars, SE.

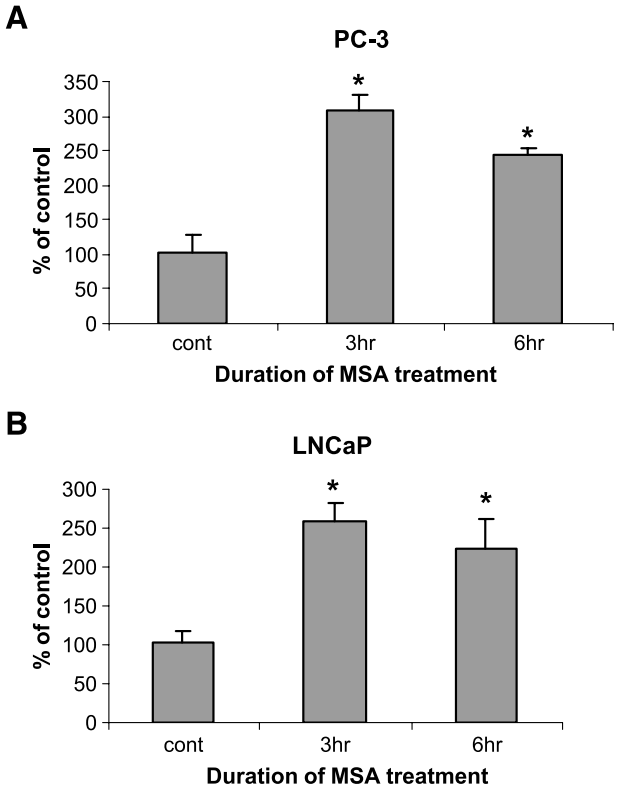

FIGURE 3. Effect of MSA on KLF4 transcription initiation as assessed by real-time RT-PCR of nascent RNA obtained by run-on transcription. Columns, mean percentage of control; bars, SE. *, $P < 0.01$, statistically different from the untreated control.

increase in transcription initiation. The contribution from increased mRNA stability seems to be relatively minor.

Gene transcription is generally controlled by promoter regions. Because major regulatory sequences are usually located near the basal transcriptional machinery, we analyzed the effect of MSA on the activity of a KLF4 proximal promoter. Cells were transiently transfected with a luciferase reporter gene construct containing 1 kb of the proximal promoter region and 550 bp of the 5′-untranslated region of the *KLF4* gene. The reporter gene assay was done during a 16-h time course. MSA treatment did not lead to any significant change in the activity of this proximal KLF4 promoter (data not shown). We are in the process of conducting further upstream cloning to identify the region responsible for MSA up-regulation of KLF4.

MSA Induces the DNA-Binding Activity of KLF4 in Prostate Cancer Cells

To determine whether increased KLF4 would lead to increased DNA-binding activity, we did electrophoretic mobility shift assays (EMSA) by using a KLF4-consensus element as the probe with nuclear extracts from PC-3 or LNCaP cells treated with 10 μmol/L of MSA for 3 h. As shown in Fig. 4A and B, an increase of KLF4-DNA complex formation was evident in MSA-treated samples when compared with that in the untreated control. The specificity of the complexes was verified by a competition analysis with an excess amount of unlabeled wild-type or mutant KLF4-consensus element as a competitor in EMSA. We then conducted a supershift assay to further demonstrate the presence of KLF4 in the complexes. As shown in Fig. 4C, incubation of the nuclear extracts with an anti-KLF4 antibody, which binds to KLF4 in a nondenaturing condition, supershifted the uppermost complex. The complexes were not affected by the addition of an anti-Ets1 antibody, which served as a negative control.

Knock-Down of KLF4 Attenuates the Effect of MSA on the Modulation of KLF4 Target Genes

Our previous microarray analysis of PC-3 cells identified three KLF4 target genes which were modulated by MSA (33). These genes are cyclin D1 (0.6), p21/WAF1 (3.5), and p27/Kip1 (2). The number in parentheses denotes the treatment to control ratio. A value of <1 or >1 signifies a decrease or an increase, respectively. To delineate the role of KLF4 as a mediator of these MSA-modulatable genes, we transiently transfected PC-3 cells with a Stealth KLF4 short interference RNA (siRNA) duplex to knock down KLF4 expression. As shown in Fig. 5A, the siRNA markedly inhibited KLF4 mRNA expression, not only in the basal condition, but also when cells were treated with MSA. The response of the KLF4 target genes to MSA was determined by real-time RT-PCR in the KLF4 knockdown cells or the scrambled control siRNA-transfected cells. MSA treatment resulted in a down-regulation of cyclin D1, and an up-regulation of p21/WAF1 and p27/Kip1 in the scrambled control-transfected cells (Fig. 5B, C and D). KLF4 knockdown significantly muted the effect of MSA on the expression of these genes.

KLF4 Up-Regulation Contributes to MSA-Mediated Growth Inhibition

In an effort to evaluate the biological significance of KLF4 up-regulation by MSA, we assessed the response of the KLF4 knockdown cells to MSA-mediated DNA synthesis inhibition and apoptosis induction. BrdU ELISA and Cell Death ELISA were conducted at 16 h post–MSA treatment. In the absence of MSA, DNA synthesis and apoptosis were not altered by KLF4 knockdown (data not shown), probably due to the relatively low basal level of KLF4 in the cells. MSA treatment inhibited DNA synthesis by >85% in the scrambled control-transfected cells, as opposed to 65% in the KLF4 knockdown cells (Fig. 6A). The difference is statistically significant ($P < 0.01$). Additionally, as presented in Fig. 6B, MSA-induced apoptosis was markedly muted as a result of KLF4 knockdown.

As a flip side to the knockdown experiment, we transiently transfected PC-3 cells with a KLF4 expression vector, and assessed the response of KLF4-overexpressing cells to MSA by BrdU ELISA and Cell Death ELISA. With the treatment of 5 μmol/L of MSA for 16 h, DNA synthesis was suppressed by 37% and 50% in the mock-transfectants and the KLF4-tansfectants, respectively (Fig. 7A). The difference is statistically significant ($P < 0.05$). In the absence of MSA, KLF4 overexpression did not affect DNA synthesis (data not shown), but significantly induced apoptosis (Fig. 7B, first two columns). The effect of MSA on apoptosis was enhanced by ~60% as a result of KLF4 overexpression (Fig. 7B, last two columns). We

lowered the dose of MSA from 10 to 5 μmol/L for the KLF-transfection experiment in order to leave room to detect an enhanced effect of MSA by KLF4 overexpression. When taken together with the knockdown data, our results showed the important role of KLF4 up-regulation in mediating the effect of MSA on growth inhibition and apoptosis.

Discussion

In this report, we present three lines of evidence to support the role of KLF4 in mediating the effect of selenium on growth inhibition and apoptosis induction in prostate cancer cells. First, selenium treatment leads to a rapid induction of KLF4 expression and DNA-binding activity. Second, KLF4 knockdown by siRNA significantly diminishes the responsiveness to selenium with respect to DNA synthesis inhibition, apoptosis induction, and the expression of three KLF4 target genes, cyclin D1, p21/WAF1, and p27/Kip1. Third, overexpression of KLF4 enhances the DNA synthesis suppressive and proapoptotic effects of selenium. It is important to put into perspective that KLF4 is one of several transcription factors of which the expression is known to be modulated by selenium at early time points of treatment. Included in this group of transcription factors are GADD153, androgen receptor, FOXO, FOXA1, ATF6, XBP1, and nuclear factor-κB (33, 36-43). Although each is reputed to regulate a different spectrum of downstream targets, a common denominator among the targets is their involvement in controlling cell proliferation and apoptosis. Thus, it is not surprising to find that knocking down KLF4 alone may not completely block the effect of selenium on growth suppression.

Other members of the KLF family may also contribute to the action of selenium. To date, 21 proteins in the KLF family have been identified in humans (44). They share a highly conserved carboxyl-terminal zinc finger DNA-binding domain and a similar DNA-binding consensus element (44). Some of these proteins contain identical DNA-interacting amino acids, and therefore, bind to the same DNA element (44). Our EMSA study with a KLF4-consensus element showed an increase in the formation of three specific DNA-protein complexes as a result of selenium treatment, whereas only one of the complexes could be supershifted by the KLF4 antibody. Because Sp1 has been shown to compete with KLF4 for DNA binding (44), we conducted supershift assays with a Sp1 antibody. However, none of the complexes were affected by the addition of the Sp1 antibody (data not shown). Our microarray data set shows that KLF5 is also increased 2- to 3-fold by selenium (33). KLF5 has been reported to be frequently down-regulated and functions as a potential tumor suppressor in prostate cancer (45). Hence, the possible involvement of KLF5 in the action of selenium deserves further investigation.

In the present study, we showed that selenium down-regulates the expression of cyclin D1, and up-regulates the expression of two CDK inhibitors, p21/WAF1 and p27/Kip1. The combined effect of selenium on these key cell cycle regulators is consistent with its activity in blocking cell cycle progression. Knocking down KLF4 in selenium-treated cells restores the expression of these genes to their control levels. In contrast, KLF4 knockdown in the untreated cells fails to bring about any changes. It is possible that in the basal condition, the transcription of these genes in prostate cells is under the control of a number of transcription factors. Knocking down one particular transcription factor would not be sufficient to significantly affect their expression. With selenium treatment, KLF4 may assume a more dominant role in the regulation of these genes.

The role of KLF4 in growth regulation is cell type– and cell context–specific (15, 16). It could either inhibit or promote cell

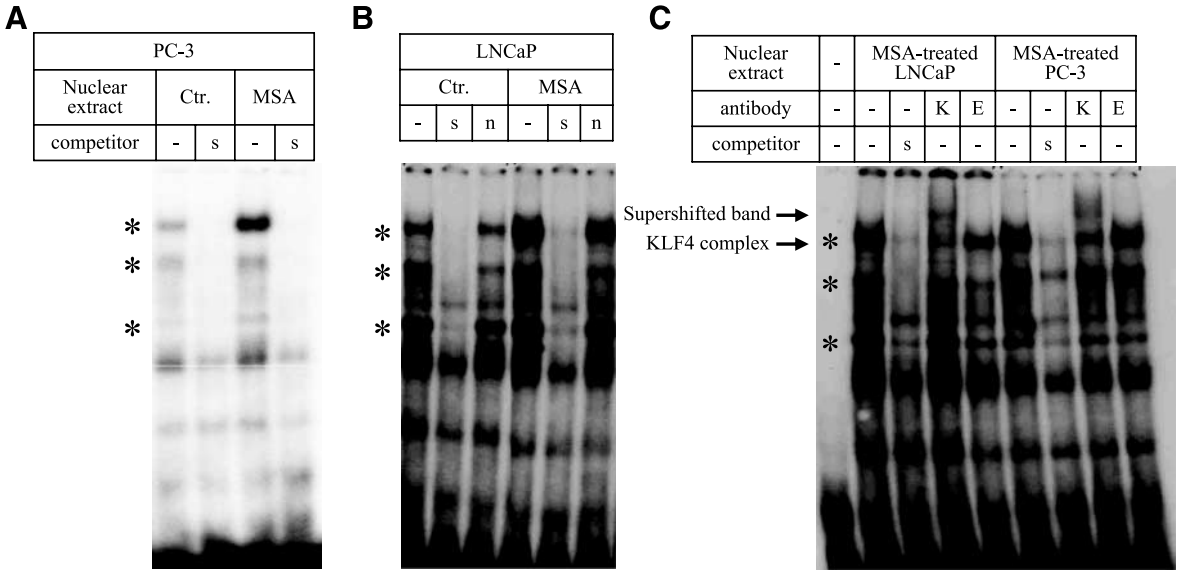

FIGURE 4. Effect of MSA on KLF4 DNA-binding activity in PC-3 and LNCaP cells. EMSA (**A** and **B**) and supershift assay (**C**) with a KLF4-consensus element. **A.** Increase of KLF4 DNA-binding activity by MSA in PC-3 cells. **B.** Increase of KLF4 DNA-binding activity by MSA in LNCaP cells. s, specific competitor (wild-type KLF4-consensus element); n, nonspecific competitor (mutant KLF4-consensus element); K, anti-KLF4 antibody; E, anti-Ets1 antibody.

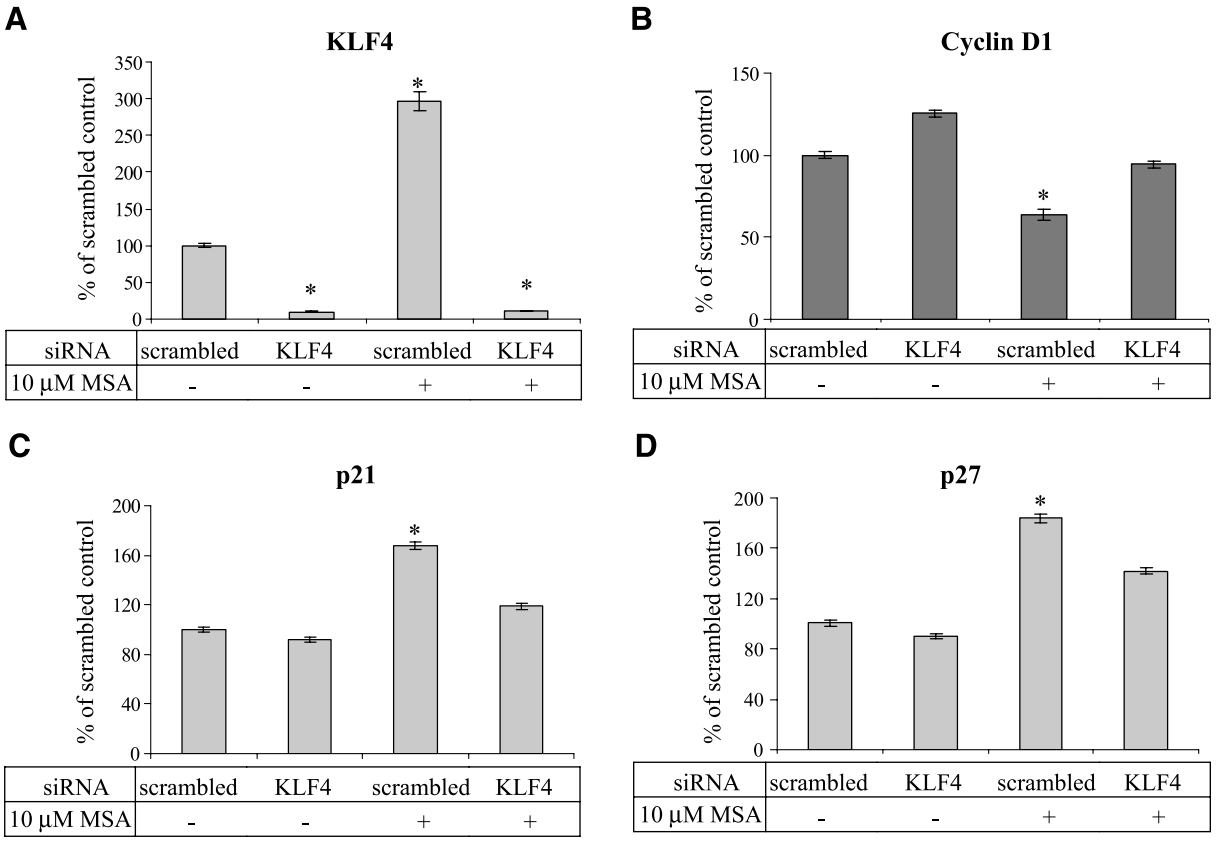

FIGURE 5. Effect of KLF4 siRNA knockdown on KLF4 target gene expression in PC-3 cells as detected by real-time RT-PCR. Columns, mean; bars, SE ($n = 3$). *, $P < 0.01$, statistically different from the untreated scrambled control.

growth (15, 16). Here, we show that transfection of KLF4 in prostate cancer cells leads to a significant induction of apoptosis. Together with the observation of decreased KLF4 expression in prostate cancer (25, 26), the data are supportive of a potential tumor-suppressing function of KLF4 in prostate cancer. Our study also suggests a proapoptotic activity of KLF4. However, none of the known targets of KLF4 have been implicated as key regulators of apoptosis. It is imperative to identify novel targets of KLF4 in order to better understand the mechanism underlying the proapoptotic function of KLF4 in prostate cancer cells.

Materials and Methods

Selenium Reagent and Prostate Cell Culture

MSA was synthesized as described previously (35). The PC-3 and LNCaP human prostate cancer cells were obtained from American Type Culture Collection. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine

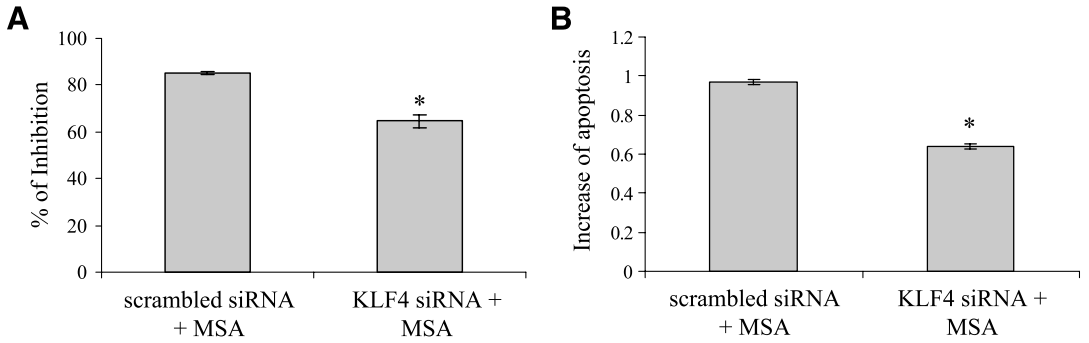

FIGURE 6. **A.** Effect of KLF4 siRNA knockdown on MSA inhibition of DNA synthesis as detected by ELISA of BrdUrd incorporation. Columns, mean; bars, SE ($n = 3$). **B.** Effect of KLF4 siRNA knockdown on MSA induction of apoptosis as detected by ELISA of DNA fragmentation. The results are expressed as an increase of apoptosis compared with the untreated control. *, $P < 0.01$, statistically different from scrambled siRNA-transfected samples.

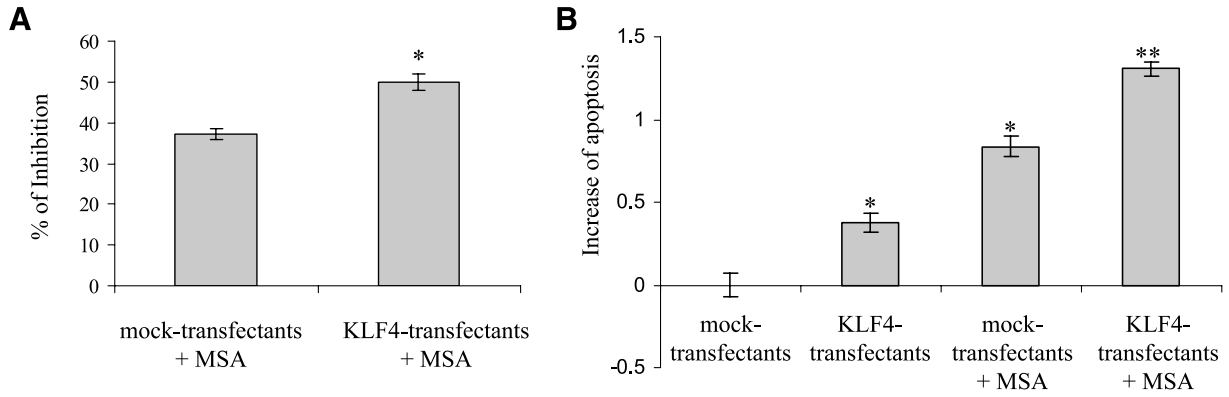

FIGURE 7. **A.** Effect of KLF4 overexpression on MSA inhibition of DNA synthesis as detected by ELISA of BrdUrd incorporation. Columns, mean; bars, SE ($n = 3$). *, $P < 0.01$, statistically different from mock-transfectants. **B.** Effect of KLF4 overexpression on MSA induction of apoptosis as detected by ELISA of DNA fragmentation. The results are expressed as an increase of apoptosis compared with the untreated mock-transfectants. MSA was present at 5 μmol/L in both experiments. *, $P < 0.01$, statistically different from the untreated mock-transfectants. **, $P < 0.01$, statistically different from the untreated KLF4-transfectants and MSA-treated mock-transfectants.

serum, 2 mmol/L of glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin.

Nuclear Lysate Preparation

Nuclear protein extracts were prepared as described previously (36). Cells were harvested, washed twice with PBS, and resuspended in a hypotonic buffer [10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, and 0.1% NP40] and incubated on ice for 10 min. Nuclei were precipitated with 3,000 × *g* centrifugation at 4°C for 10 min. After washing once with the hypotonic buffer, the nuclei were lysed in a lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, and 1% Triton X-100] and incubated on ice for 30 min. The nuclear lysate was precleared by 20,000 × *g* centrifugation at 4°C for 15 min. Protein concentration was determined by using the bicinchoninic acid protein assay kit (Pierce).

EMSA

EMSA was conducted as previously described with 10 μg of nuclear protein extract and 1 ng of end-labeled double-stranded oligonucleotide probe (46). The unlabeled oligonucleotide competitors were present at 100× in excess in the competition experiments. The sequences of the oligonucleotides used in the assays are as follows: the wild-type KLF4-consensus element, 5′-ATGCAGGAGAAAGAAGGGCGTAGTATCTACTAG-3′ (47); the mutated KLF4-consensus element, 5′-ATGCAGGAGAAAGAAGTTCGTAGTATCTACTAG-3′ (47). For the supershift assays, the antibody against KLF4 or Ets-1 (both from Santa Cruz Biotechnology) was added after the formation of the protein-DNA complexes, and incubation was continued for an additional 30 min on ice. The reaction mixtures were analyzed by 5% PAGE, and the gel was subsequently dried and visualized by autoradiography.

Real-time RT-PCR

The analysis was done as described previously (36) by using RNA isolated with the TRIzol reagent (Invitrogen). The PCR primers and TaqMan probes for β-actin (a housekeeping gene, assay ID Hs99999903_m1), KLF4 (assay ID Hs00358836_m1), cyclin D1 (assay ID Hs00277039_m1), p21/WAF1 (assay ID Hs00355782_m1), and p27/Kip1 (assay ID Hs153227_m1) were Assays-on-Demand products from Applied Biosystems. The PCR conditions were as follows: an initial incubation at 50°C for 2 min, then a denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Temperature cycling and real-time fluorescence measurement were done by using an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The relative quantitation of gene expression was done by using the comparative C_T ($\Delta\Delta C_T$) method (48). Each real-time RT-PCR experiment was done in triplicate, and the mean C_T value was used for data analysis. The final result is presented as the mean of three separate experiments ± SE.

KLF4 mRNA Stability Assay

Actinomycin D (5 μg/mL) was added to the cultures to stop new RNA synthesis at the time of MSA treatment, and KLF4 mRNA levels were measured by real-time RT-PCR at the 2, 4, and 6 h time points. The turnover of KLF4 mRNA was determined by comparing mRNA levels over time in cells treated with or without MSA.

Reporter Gene Assay

The KLF4-pGL2-luciferase reporter gene construct (49), containing 1 kb of the 5′-flanking region and 550 bp of the 5′-untranslated region of the *KLF4* gene, was kindly provided by Dr. Vincent W. Yang at Emory University. This plasmid was transiently transfected into cells with the LipofectAMINE and Plus reagents (Invitrogen) at a concentration of 4 μg per 10 cm culture dish. After incubating with the transfection mixture for 3 h, the cells were trypsinized and replated in triplicate onto six-well plates. Cells were allowed to recover for an additional 24 h before exposure to 10 μmol/L of MSA. After 1, 2, 3, 6, or 16 h of MSA treatment, cells were lysed in reporter lysis buffer (Promega), and the luciferase activity was assayed by using the Luciferase Assay System (Promega). Protein concentration in cell extracts was determined with the bicinchoninic acid protein

assay kit (Pierce). Luciferase activities were normalized by the protein concentration of the sample. The transfection experiments were repeated thrice.

Quantitative Nuclear Run-on Assay

Run-on transcription was done according to a previously described method with biotin-16-UTP (Roche; ref. 50). Biotin-labeled nascent transcripts were then isolated by using streptavidin particle beads, and quantitated by real-time RT-PCR. The experiment was done thrice in triplicate.

Transient Transfection of a KLF4 Expression Vector

The KLF4 expression vector, pcDNA3.1/His B-GKLF (51), was a gift from Dr. Anil K. Rustgi at the University of Pennsylvania. The transfection was carried out by using the LipofectAMINE and Plus reagents (Invitrogen) according to the instructions of the manufacturer. At 24 h before transfection, cells were plated in growth medium without antibiotics at a density to reach 90% to 95% confluency at transfection. The KLF4 expression vector or the pcDNA3.1/His B mock plasmid was introduced into cells at a concentration of 4 μg per 10 cm culture dish. The DNA/liposome mixture was removed at 3 h after transfection. The cells were trypsinized 16 h later and replated in triplicate onto two 96-well plates, one for BrdU ELISA and the other for Cell Death ELISA. Cells were allowed to recover for an additional 24 h before exposure to 5 μmol/L of MSA. BrdU ELISA and Cell Death ELISA were conducted 16 h post–MSA treatment.

RNA Interference

A set of three predesigned Stealth Select siRNA duplexes to the human *KLF4* gene as well as the Stealth siRNA Negative Control Duplex with medium GC content were purchased from Invitrogen. The KLF4 siRNA duplex with the highest efficacy in knocking down KLF4 expression was used for the experiment. The sequence of this siRNA (KLF4-HSS113796) was 5′-GGACCUGGACUUUAUUCUCUCCAAU-3′, corresponding to nucleotides 611 to 635 of the human KLF4 cDNA sequence (GenBank accession no. NM_004235).

The siRNAs were introduced into cells by using the LipofectAMINE 2000 reagent (Invitrogen). Transfection efficiency and optimal transfection condition were determined by using the BLOCK-iT fluorescent RNA duplex (Invitrogen), which is not homologous to any known gene. At 24 h prior to transfection, cells were plated in triplicate in growth medium without antibiotics at a density to reach 60% to 70% confluency at transfection. The siRNAs were transfected into cells at a concentration of 80 nmol/L. The cells were treated with 10 μmol/L of MSA at 24 h after transfection. Real-time RT-PCR analysis, BrdU ELISA, and Cell Death ELISA were conducted at 16 h post–MSA treatment.

Cell Proliferation Assay

Proliferation was measured by using the BrdU Cell Proliferation ELISA kit (Roche) according to the instructions of the manufacturer with minor modifications. Briefly, after labeling the cells with bromodeoxyuridine (BrdUrd) for 2 h, the WST-1 reagent (Roche), which quantitatively monitors the metabolic activity of the cells, was added to the wells to a final concentration of 10%. The cells were incubated for an additional 2 h. The amount of formazan converted from WST-1 by the metabolically active cells was quantitated at 450 nm. After removing the medium, the cells were fixed and the DNA denatured for the incorporated BrdUrd to bind to a peroxidase-conjugated anti-BrdUrd antibody. The immune complexes were detected by the subsequent substrate reaction, and the reaction product was quantified by absorbance at 370 nm (reference wavelength at 492 nm). Culture medium without cells and cells incubated with the anti-BrdUrd peroxidase antibody in the absence of BrdUrd were used as controls for nonspecific binding. The BrdU ELISA result was normalized by the WST-1 reading, which correlates directly with the cell number. The experiment was done thrice in triplicate.

Apoptosis Detection

Detached cells were precipitated by centrifugation and pooled with attached cells. Cytoplasmic histone-associated DNA fragments were quantified by using the Cell Death Detection ELISA^{PLUS} Kit (Roche Applied Science) according to the protocols of the manufacturer. The absorbance was measured at 405 nm (reference wavelength at 492 nm). The experiment was done thrice in triplicate.

Statistical Analysis

Mean activities were calculated from three independent experiments done in triplicate. The Student's two-tailed t test was used to determine significant differences between treatment and control values.

Acknowledgments

We sincerely appreciate the invaluable suggestions and assistance from Drs. Clement Ip and Adrian R. Black at Roswell Park Cancer Institute during the preparation of the manuscript. We are grateful to Dorothy Donovan, Todd Parsons, and Cathy Russin for their excellent technical assistance. We also extend our gratitude to Dr. Vincent W. Yang (Division of Digestive Diseases, Department of Medicine, Emory University, Atlanta, GA) for the generous gift of the KLF4-pGL2-luciferase plasmid and to Dr. Anil K. Rustgi (Division of Gastroenterology, Department of Medicine, University of Pennsylvania, Philadelphia, PA) for kindly providing the pcDNA3.1/His B-GKLF construct.

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